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A FURTHER STUDY OF BAR-EYED MOSAICS IN DROSOPHILA

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(Received for publication, October 19, 1942)

It has long been recognized that genetic factors which control the size of a part of the organism with little or no change in the rest of the morphological pattern make favorable material for the study of how the genes act to produce their effects. Such a series in Drosophila is the bar series of eye reducers. At 25° *C*. this reduction is from about 750 facets for the wild type down through baroid, infrabar, several bars, double infrabar, ultrabar, on down to quadruple bar, the lowest member of the series, with about 10 facets or less in the compound eye. Data pertinent to the problem have been gathered by a number of investigators since Zeleny began the work more than 25 years ago. This work has included studies on the effects of temperature and of oxygen pressure, transplantation studies, various feeding and starvation experiments, and studies on mosaic eyes, which involve a change at the bar locus during development.

In a previous study of white bar-eyed mosaics (Hersh and DeMarinis, 1938) caused by somatic segregation in flies heterozygous for a member of the bar series and also for the genes for white eye and Minute-n bristles, it was found that the relation between the number of white facets (y) and the total number of facets (x) in the mosaic eye conformed as a rough approximation to the relative growth function

$$y=bx^k$$

In logarithmic form the equation becomes

$$\log y = \log b + k \log x$$

Consequently when the data are plotted on a logarithmic grid the result is a straight line, actually a band with parallel edges, with slope k and y-intercept b.

In the study mentioned, the value of k was nearly 4, irrespective of the genetic constitution of the flies or of the temperature at which they were raised. The constant b was the one chiefly affected.

After somatic segregation in flies of the constitution *fBMn/w*, the

resulting cells have the constitution *ſBMn/ſBMn* and *w/w*. The former of these presumably die, leaving cells with a genetic constitution for white eye, wild type at the bar locus, in an optic disk which is otherwise *ſBMn/w* in constitution. In the mosaics mentioned the change at the bar locus was for an increase in size of the eye, with the exception of one series in females of the constitution *ſBMn/wBB*, in which the change at the bar locus was for a smaller number of facets. In this series of mosaics the chief effect was still on the constant *b*, although the value of *k* was reduced to slightly less than 3.

The purpose of the present paper is mainly to present data on eight more sets of mosaics and to discuss the quantitative relations when the change at the bar locus is for a smaller eye. The present data include also mosaics for another member of the bar series, namely *BiBi*.

The data are given in Table 1. For each fly, under the genetic constitution and the temperature indicated, there are given the number of white facets and the total number of facets in the mosaic eye; also the total number of facets in the opposite eye. The other eye in a few cases was entirely white, indicated in the table by (*w*). The few cases with a mosaic eye and an all white opposite eye are no doubt the result of a single event, either a chromosome elimination or a somatic segregation, which occurred early enough to affect both eyes. The number of mosaics of Table 1 and of the previous paper (Hersh and DeMarinis) give a combined total of 200 mosaics recovered from 25,977 Minute-n females.

In the genetic constitutions in Table 1, there is listed the constitution of the *X*-chromosome contributed by the mother followed by that of the father. Since somatic segregation is the result of crossing over which in the present case may be taken as always to the right of Minute-n (locus 62.7), it follows that the crossing over is also to the right of the bar locus (57.0). Consequently, the white spot in the mosaic eye is diagnostic of the change at the bar locus and this change may be for a larger or a smaller eye, depending upon the composition of the *X*-chromosome contributed by the male parent. After the somatic segregation occurs, the spot is homozygous for the paternal *X*-chromosome in a fly which is heterozygous for one of the respective genetic constitutions given in Table 1. For each series of mosaics the facet counts are arranged according to increasing number of white

TABLE 1

For each fly, under the genetic constitution indicated, there are given the number of white facets and the total facet number in the mosaic eye, followed by the number of facets in the opposite eye.

Mn/wfBB at 25°C.		
1	55	51
1	41	41
1	47	48
1	46	45
3	28	37
4	33	46
5	30	40
5	34	33
7	39	40
11	34	31(w)
15	20	46
22	27	44
24	24	20(w)

Mn/wfB at 25°C.		
1	166	178
1	205	170
1	183	186
1	155	161
2	244	256
2	287	283
2	279	278
3	117	137
4	356	375
4	240	269
4	151	170
4	222	248
6	331	376
6	130	132
11	430	416
12	175	228
21	330	367
27	180	182
43	260	281
73	186	198
83	408	397

Mn/wfB1B1 at 20°C.		
1	67	77
1	80	70
1	96	95
1	90	91
1	64	72
2	73	72
2	90	95
2	114	106
2	78	78
2	74	70
2	95	86
2	79	72
3	56	62
3	81	80
4	88	75
4	75	73
4	90	84
5	94	90
5	93	92
6	66	71
7	108	92
9	103	87
17	91	82
17	88	77
19	65	84
21	88	104
33	92	96
51	53	80

Mn/wfBB at 20°C.		
1	40	45
1	47	45
1	37	43
1	50	55
9	40	49
18	37	46
32	32	47

Mn/wfB at 20°C.		
1	378	372
3	386	389
3	280	296
4	384	407
4	311	310
5	293	278
6	356	322
7	240	268
9	384	411
10	279	329
10	236	249
11	268	229
14	206	228
15	288	325
18	316	327
18	320	274
20	332	287
38	249	120(w)
70	125	258
237	258	210(w)

yB1B11n/w at 25°C.		
1	289	264
2	252	240
4	321	340
5	295	258
6	245	205
9	270	275
10	385	360
17	512	522
23	308	337
25	297	302
36	398	354
37	268	204
49	429	457
284	481	264
285	653	375
797	802	809(w)

yB1B11n/w at 20°C.		
3	362	364
4	380	350
4	348	322
5	334	332
5	392	357
6	380	391
9	440	444
10	307	258
11	337	323
13	383	343
14	402	327
15	343	320
16	364	309
25	507	468
36	412	336
44	421	330
47	405	364
48	539	393
70	472	367
77	450	744(w)
84	461	349
156	600	345
230	597	340
775	775	729(w)

Mn/wfB1B1 at 25°C.		
1	98	105
3	32	42
3	60	- -
5	90	82
7	63	- -
9	53	- -
10	111	94
15	104	106
28	65	- -
37	95	101
50	62	85

facets in the mosaic spot. A cursory examination shows that, although there is considerable variability, yet on the whole for some series as the white facets increase in number the total number of facets in the mosaic eye increases, other series show a decrease in total eye size as the number of white facets increases and there are others which show no such relation. Each series will be discussed from the standpoint of the relative growth equation.

In the mosaics of the constitution *yBiBiMn/w* at 20° and 25° the change at the bar locus is for a larger eye. When the number of white facets (y) is plotted logarithmically against the total number of facets (x) in the mosaic eye, the result in each case is a narrow band of points. When the data are grouped and the constants calculated by the method of averages, it is found that k has the value 5.4 for the mosaics at 20° and 5.62 for those at 25° (Figure 1, *G* and *H*). The curves are sensibly parallel to one another and to the curves for the mosaics in the *fBMn/w* series at 20° and 25°, which are included in Figure 1, *F* and *E* (see Hersh and DeMarinis, 1938). In all four cases the change at the bar locus is to the wild type condition in flies heterozygous for the member of the bar series indicated. It seems clear that as the somatic segregation occurs earlier and earlier during development, resulting in a larger and larger white spot, that the growth processes in the optic disk which produce more white facets lead finally to an entirely white eye of the same order of size as the wild type or somewhat smaller, depending upon the temperature. The average value of k for the four curves is about 4.5. In other words, with increasing eye size the number of white facets increase about 4.5 times faster than total facet number.

In the other sets of mosaics of Table 1 the change at the bar locus was for a smaller eye than that for the genetic constitution of the flies indicated. Although again there is considerable variability, yet three of the sets conform to the relative growth equation with negative values of k. These three sets are the *Mn/wfBB* at 20° and 25°, for which the values of k are, respectively, —12.04 and —2.96 (Figure 1, *C* and *A*), and the *Mn/wfB* set at 20°, for which the value of k is —1.84 (Figure 1, *D*). In other words, for these three sets of mosaics, as the number of white facets increases the total number of facets decreases, as one might predict from the knowledge that the change at the bar locus is for a smaller eye. In the *Mn/wfBB* set of mosaics at 20° the value of k is such that the regression line

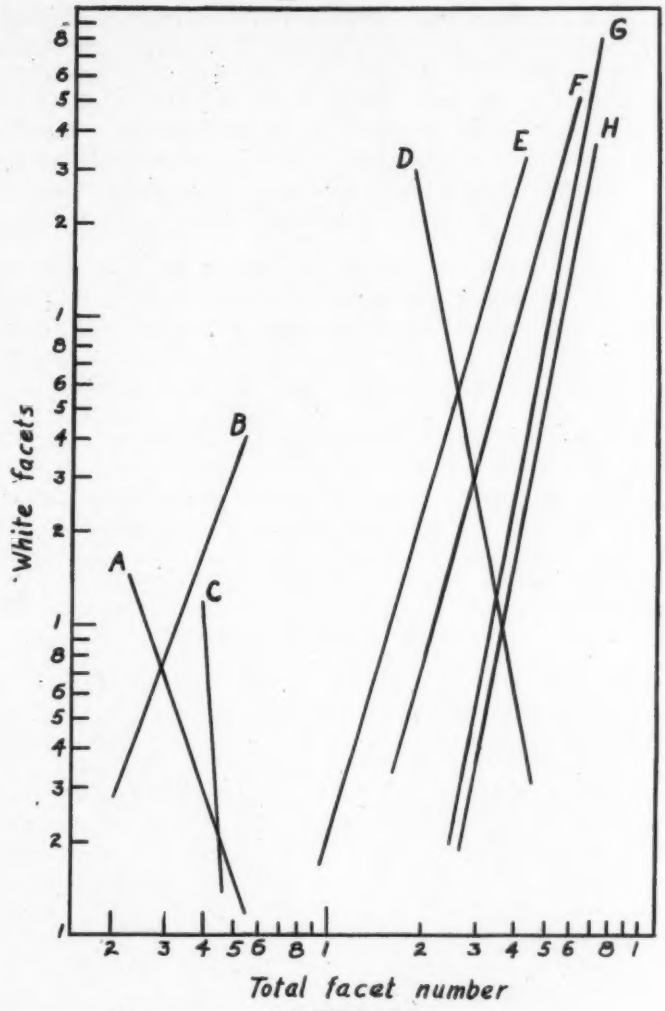

FIGURE 1

FITTED LINES FOR THE DATA ON VARIOUS SETS OF MOSAICS

The number of white facets (y) is plotted logarithmically against the total number of facets (x) in the mosaic eye. *A*, Mn/wfBB at 25°; *B*, fBMn/wfBB at 25°; *C*, Mn/wfBB at 20°; *D*, Mn/wfB at 20°; *E*, fBMn/w at 25°; *F*, fBMn/w at 20°; *G*, yBiBiMn/w at 25°; *H*, yBiBiMn/w at 20°. (*B*, *E*, and *F* are taken from Hersh and DeMarinis, 1938.)

(Figure 1, *C*) departs only slightly from the perpendicular position. It is this series which gives an indication of the transition from the other sets to the three sets still to be discussed. For each of these three sets, namely, the *Mn/wfB* set at 25° and the *Mn/wfBiBi* sets at 20° and 25°, the band of points is practically parallel to the *y*-axis. In terms of the relative growth equation the values of k tend to infinity. In terms of statistical correlation, the regression coefficient likewise tends to infinity and the coefficient of correlation (r) does not differ significantly from zero.

The experience from the 11 sets of mosaics leads then to the conclusion that the somatic segregation in relation to the two main factors which affect eye size, namely, genetic constitution and temperature of development, may give values of k from a low positive value somewhat above 1 to larger positive values which pass through infinity to negative values of k for those sets of mosaics which involve a change at the bar locus for a smaller eye, down to small negative values of k in the neighborhood of —1.

The differences in the values of k seem clearly to be related to growth processes in the optic disk prevailing at the time the somatic segregation occurs and depending upon whether it brings about a change which exerts a positive or negative effect on the growth of the optic disk.

General Discussion

The rôle that members of the bar series play in the developmental nexus to bring about the marked defect in the size of the eye without marked change in the rest of the pattern needs to be linked with the main processes which occur during development. The processes we have chiefly in mind are embryonic determination and growth, although to be sure beyond these are the many interlocking biochemical and physiological processes which occur throughout development.

While no one claims that obviousness is a necessary guarantee of the truth, yet it seems entirely certain that whatever rôle the members of the bar series play, they are intimately related to the complicated events of growth during the immature stages of development from the egg to the adult stage. The marked minus effect in the size of the eye, or any localized dwarfism, may be brought about in three different possible ways. First of all a normal embryonic process may occur resulting in a normal sized anlage, from which at a later stage there is an actual loss of tissue by resorption, autolysis, atrophy, or

autotomy; secondly, the processes leading to a normal anlage may be defective, so that the localized dwarfism is not an actual loss but only an apparent loss because of an agenesis of material in the mutant which is produced in the normal or wild type. A third way for the production of a localized dwarf effect is typified by miniature wing in Drosophila. In this case the normal number of cells is produced but the cells fail to undergo the normal increase in size. In any particular case these three different ways may, of course, act alone or together.

Since the facets of the eye in the bar series are normal in size, it is clear that the eyes for the bar series are not like miniature wing, the third type of arrested development mentioned above. The data on mosaics favor the view that in members of the bar series, the eye is small because of an agensis of tissue which is produced in the wild type. When, for example, the somatic segregation brings about a change at the bar locus for a larger eye, then the larger eye is a reflection of the increased growth, resulting in a larger optic disk, or at any rate of that part of the disk which develops into ommatidia. And the earlier the change takes place the greater the amount of increased growth. On the other hand, when the genetic change at the bar locus is for a smaller eye, then the opposite is the case, i.e., after the change there is less growth in the optic disk, and the earlier change takes place, the greater is the defect in the size of the optic disk. Although the eye is smaller yet it contains relatively more white facets. This interpretation is graphically presented in Figure 2.

The continuous curves represent the growth curves of the optic disk, or perhaps better, the part of it which becomes the eye. The curve for the wild type extends throughout the three larval stages, while those for bar and ultrabar, *B/B* and *BB/BB*, respectively, are confined to the third larval stage, an interpretation which takes into consideration the position of the temperature effective period at standard temperatures. The curve for heterozygous bar, *B/+*, has a dashed portion leaving it on the upper side and one on the lower side. These represent, respectively, the increased growth when the change at the bar locus is for a larger eye and the decreased growth when the change is for a smaller eye. The tissue which is proliferated during these dashed portions of the curve will later produce the white facets in the mosaic eye. If the genetic change is for a larger eye, and it happens early enough, then the eye will be entirely white and some-

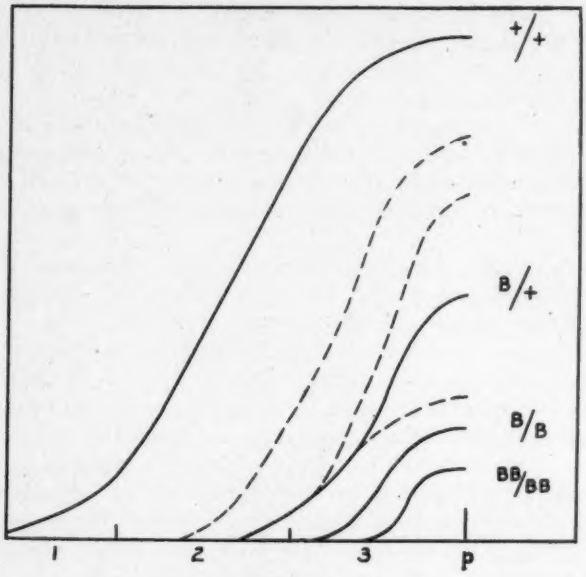

FIGURE 2

HYPOTHETICAL CURVES OF THE GROWTH OF THE OPTIC DISK, OR OF THAT PART OF IT WHICH BECOMES OMMATIDIA

Along the time axis 1, 2, and 3 represent the larval stages; *p*, the time of pupation. +/+, wild type; *B*/+, heterozygous bar; *B*/*B*, homozygous bar; and *BB*/*BB*, homozygous ultrabar. (See text for discussion.)

what smaller than the wild type. Such a change is shown in the figure by the curve dashed throughout its whole extent. The underlying idea of the figure is to give a series of curves which represent not only the case of the bar series but one which is more or less general for any case where the localized dwarfism is an agenesis characterized by progressively later times of onset for the growth of the part, and so stands in contrast to the figure contributed by Goldschmidt and Höner (1937, Figure 2) for the case of the vestigial wing series, which involves an actual loss from the morphological pattern.

Since in the case of the bar series, the change in growth results in an increase or decrease in the final number of cells, the further conclusion seems justified that the bar factors are intracellular agents which control the elaboration of an activator for cell division in this

part of the body of the fly. The data throw no light on the problem of how it happens that the effect is localized in the optic disk.

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A STUDY OF FOUR GENERATIONS OF RATS TREATED WITH LARGE AMOUNTS OF GLUTATHIONE*

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Since the original reports by Asher and Ratti (1) and by Rowntree and his associates (2, 3) that extracts of thymus glands injected into rats produced accelerated growth and development in successive generations, many unsuccessful attempts have been made to confirm their findings. Rowntree and his associates have published results showing that glutathione is not only present in large quantities in thymus extracts, but that glutathione possesses the same growth promoting properties as the thymus extracts (4, 5, 6). Thus the conclusion was reached that glutathione was the active substance in the extracts. To our knowledge, no one has ever been able to confirm the results of either group of investigators. The studies of Segaloff and Nelson (7, 8) are probably the most elaborate and careful efforts to confirm the work of Rowntree thus far published. The results were negative, but a partial (at least) explanation for the results of the earlier experiments of Rowntree was offered. It was suggested by Segaloff and Nelson that very probably dietary deficiencies were responsible for the failure of Rowntree's control animals to grow at the same rate as his experimental animals. At any rate, with adequate diet and care, Segaloff and Nelson could find no differences between animals injected with thymus extract or glutathione and the controls carried through five generations. Similar results have been reported for thymus extract by Neuwirth and Venokur (9).

During the past two years a somewhat similar study has been under way in this laboratory, the results of which we wish to present at this time.

MATERIALS AND METHODS

Twenty-six male and 20 female rats from the University stock col-

*A part of the expenses of this investigation were borne by a grant from the Nutrition Research Laboratories.

Grateful acknowledgment is made to Works Project Administration, Project No. 30552, for technical assistance.

ony were used. This colony originated from the Sprague-Dawley Strain, but has been maintained at the University of Illinois for several years. The growth rate and other characteristics of the colony are very uniform and compare favorably with other colonies. The animals were divided equally into control and experimental groups, littermates being divided equally between the two groups. The experiment was started at weaning (21 days). Experimental animals received daily intraperitoneal injections of 25 *mg* of glutathione dissolved in 0.5 *cc* of saline. Control animals were injected with the same volume of saline. The solution of glutathione was prepared daily from crystalline glutathione and sterile saline. The glutathione was prepared from fresh bakers' yeast, according to the method of Schroeder, Collier, and Woodward (10).

At 100 days of age the rats were mated. The injections were continued throughout pregnancy and lactation, and at weaning the young were subjected to the same treatment. This was continued for four generations. The diet used was the stock diet of the colony: Purina Fox Chow and tap water, supplemented twice weekly with lettuce or carrots. During pregnancy and lactation the females were given milk *ad lib.* and the young were also given milk for 20 days after weaning.

Weekly records were kept of body weight, body length of each rat, and the date of eruption of the incisors was noted.[1]

Results and Discussion

It will be noted that the dose of glutathione used in these experiments was 25 *mg.* daily for each rat. The dose was not changed as the weight of the animals increased. Thus the amount of glutathione each rat received was extremely large in comparison with that given by Segaloff and Nelson when the rats were in their most rapid stage of growth. It was felt that if glutathione would produce any acceleration of growth, the large dose should make the effect more obvious. It is extremely doubtful that this amount of glutathione could produce any toxic effects.

Taken as a whole the results were negative. There was no acceleration of growth in the first, third, and fourth generation. In the case of the males of the second generation, there was an apparent decrease in weight of the experimental as compared with the control animals

[1]Dates of eruption of incisors were noted by Fred Herzberg, D.D.S. of the College of Dentistry of the University of Illinois.

during the first 150 days of life, but later the two curves tended to approach each other, so that at 230 days of age the difference was no longer significant. No explanation can be offered for this effect which was not found in the littermate females undergoing the same treatment. The weights of the animals of each generation at 100 days of age are shown in Table 1, and the growth curve for the males of the

TABLE 1

WEIGHTS OF CONTROL AND EXPERIMENTAL ANIMALS AT APPROXIMATELY 100 DAYS OF AGE

Generation	Sex	Control		Experimental	
		Wt. (gm)	No. of rats	Wt. (gm)	No. of rats
1	♂	309±24	11	290±32	13
	♀	201±16	10	191±14	10
2	♂	312±25	5	258±15	5
	♀	205±20	5	190±25	5
3	♂	360	3	357	3
	♀	229±11	6	217	2
4*	♂	223±20	6	242±20	5
	♀	172±10	5	178	3

*At age of 65 days. Experiment concluded.

second generation are shown in Figure 1. Data for body length and date of eruption of incisors are not included, since the difference between control and injected animals is even less than for body weight. The number of animals in the third and fourth generations was in some cases smaller than desirable for this type of study, but for some unknown reason, neither the control nor the experimental animals were as successful breeders at this stage as during the earlier generations.

In addition to measuring weight, body length, and date of eruption of incisors, the skeletal muscle, liver, and kidneys of each rat were analyzed for reduced and total glutathione, and skeletal muscle was also analyzed for total acid soluble phosphorous. The glutathione determinations were made by the iodometric method of Okuda and Ogawa (11). The results are not included since they showed no differences between control and experimental animals in any generation.

The results presented here lend strong confirmation to those of Segaloff and Nelson (8), and are further evidence that glutathione is not a growth promoter for the albino rat. Taken in conjunction with their results on thymus extract, it appears that neither glutathione nor thymus extracts can be responsible for the results obtained by Rowntree and his collaborators.

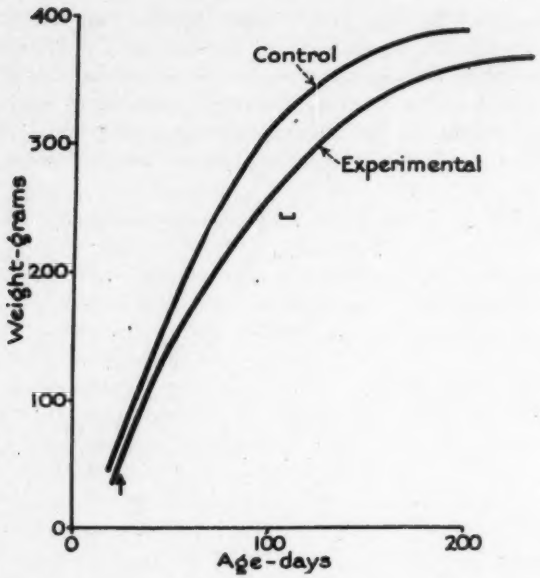

FIGURE 1

GROWTH CURVES OF MALE RATS OF SECOND GENERATION

(Administration of glutathione started at arrow. ⊔ indicates period of mating.)

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THE GEOMETRICAL BASIS OF BIOLOGICAL PROPERTIES

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The systematization of biological properties has been undertaken by Sleggs ('39, '40, '41) under the heading of *the theory of differential periodicity*. This hypothesis, moreover, extends to the actual pattern of the organism. The theory of differential periodicity is the first general theory of the organism, for others are concerned with localized phenomena or special aspects of organic behaviour. It is, further, the only biological theory whose designation has been, or can be, reduced to a mathematical principle. Fortunately, from the standpoint of biologists, the theory requires no mathematical knowledge, for it can be understood by the inspection or visualization of geometrical figures, and, while it is actually capable of mathematical treatment and development its application to genetics and growth studies can be effected by measurements upon such constructions. The term periodicity is used in the spatial sense, differential periodicity meaning differential spacing or different spacings. Such differential spacing is postulated in the megachemistry of protoplasm and particularly in the structure of the gene column, in which lattices are superposed or interpenetrated at various inclinations. The organism is regarded as a stereometric pattern worked out by the interplay of molecular rhythm. The wave field concept so reached serves as an explanatory tool, for the properties of wave systems are already extensively known and are thus available for purposes of biological interpretation. It is the pattern of such a system, which varies according to the inclinations and translations of the component lattices, which is the pattern of the organism; and the general properties of it which constitute what is termed life.

If these claims are true, let us contrast the position with what obtains in the absence of such a theory. There exist at the present time a number of impressive treatises by biological specialists. These take the form of exhaustive summaries of research in some field of biology and are accompanied usually by the author's own theory in relation to the particular and frequently the general subject. But one observes that there is little unification among them, even if the conclusions do

not directly conflict. The works which the writer has in mind will be stated, with brief notes as to their contents.

1. Darlington ('37) *Recent Advances in Cytology;* ('38) *Evolution of Genetic Systems.* Deals specifically with chromosomes, other cellular properties receiving slight treatment. Main thesis is that nuclear events are extensively subject to accidents, supporting theory of fortuitous evolution. Maintains that selection is only basis of a rational biology and that a system in which biological properties originate as inherent faculties is inconceivable.

2. Gray ('31) *Experimental Cytology.* Deals with physical and chemical properties of protoplasm. Little reference to cytogenetics: hardly any of Darlington's facts are in Gray and vice versa. Author markedly non-committal in conclusions, emphasizing difficulty and disheartening nature of problem. Tentatively suggests key to life is in circumvention in some unknown way of second law of thermodynamics; or in some form of orientation of protoplasmic molecules not yet conjectured.

3. Huxley ('32) *Problems of Relative Growth.* Treats exhaustively of growth gradients. Establishes exponential law of growth partition, according to which size of parts is controlled both in development and adult. Considers late animal growth, early animal growth, and plant growth subject to different sets of laws. Takes view that genic effects are superposed on more fundamental growth mechanism; elsewhere that genes might also be basis of latter. Considers growth gradient data consistent with Goldschmidt's theories. Regards basis of gradient obscure. No suggestion regarding the figure of the organism.

4. Goldschmidt ('38) *Physiological Genetics.* Advances theory of rate processes. Gene controls rate of formation of a substance (growth promoting, inhibiting, lytic, etc.). Explains a number of specific patterns with extensive aid of subhypotheses and on basis of previous pattern. No suggestion regarding figure of organism. Considers random factors exist in morphogenesis; nevertheless development has high precision.

5. Dürken ('29) *Experimental Analysis of Development.* Considers gene theory unsatisfactory in general effect and in detail. Follows Driesch in view that organism is not wholly mechanistic. Develops concept of specific organismic faculty.

6. Dalcq ('38) *Form and Causality in Early Development.* Derives differentiation largely from cytoplasmic gradients in the egg. Little

reference to genes. While mechanistic, conveys insinuation (as Dürken) that problem of the organism is to be explained largely by looking at it in the right way.

Further examples of differing viewpoints and more pointed controversy could be readily multiplied. Gulick ('39) considers that the chromosomes must be the sole basis of heredity, although he also points out that they do not possess chemical continuity. Harvey ('36) says "No particular type of visible and moveable granules seems essential to development. It must therefore be the ground substance which is the material foundation for development which . . . in the living egg is optically empty."

An observer of this volume of controversy and unintegrated fact will probably conclude that the problem is one which cannot be treated by any process of direct and continuous reasoning: it is one not for the intellect but the power of conjecture. Its solution may be simple but difficult to think of. Some of the most enigmatical problems have this peculiarity, as witness conjuring tricks and mechanical puzzles. A single sentence may suffice to convey the complete explanation of the most bewildering and spectacular display of magic, and it is here where the theory of differential periodicity steps in, for the gist of it is this: *life is the expression, by matter and energy, of the properties of a figure consisting of systems of evenly spaced points.* No limitations are, of course, stipulated in this definition, so that the figure must be thought of as a system of interpenetrated components at all possible angles. The points come variously into association so as to form a pattern of rarefaction and condensation which has the form of a wave field.

One may ask at this stage, if the theory is right, why has not the principle of differential periodicity previously received consideration in a biological connection, for it is well known in physical systems and in pure mathematics, where different periodicities are frequently superimposed. Nevertheless the fact is that it has received practically no consideration. The writer knows of one case where it has been applied to the direct interpretation of pattern, namely in the cell wall of *Valonia ventricosa,* by Preston and Astbury ('37), who refer to "a multiple structure . . . with extinction positions not coincident." It has been applied indirectly to the crimp in wool by Barker and Norris ('30) who postulate growth forces operating perpendicularly in the hair follicle. In protein chemistry the principle is involved, though not in

the specific form of the present theory, in Bergmann and Niemann's Law according to which each aminoacid residue occurs at regular intervals in the polypeptid chain but with different frequencies, which has been criticized by Astbury ('37) on the grounds that this would lead to coincidence of different residues.

Wave systems are usually considered in the form of a succession of ridge-like crests, or undulations in a single line, but this is a special case of periodicity. The general case is where the periodicity is represented in two dimensions and the periodic component is a set of evenly spaced peaks. It is this form of periodicity which it is essential to consider in relation to the organism, for the first does not lead very far. While Haldane has suggested that wave mechanics may find some application in biology, the fact is that biologists have not been wave-minded. I do not believe the word wave or wave field occurs in Huxley, although such a concept would immediately clarify his gradient fields. Goldschmidt does not postulate a rhythm in his genic rates, although this would relieve his theory of the need of continually supposing thresholds. There has, from the earliest period of biology, always existed a school of thought which maintains that the pattern of the organism is produced on a basis of architecturality and not by the loose diffusions of substances which must be supposed to form organs in the fashion of Liesegang rings or as clouds in the sky are formed by the interaction of atmospheric circulations. The chief consideration which supports this view, which may be termed the crystallographic view, is the high precision of differentiation, which is inconceivable in any loose system, and especially when the latter is subject to quite violent influences arising from the internal circulations and movements of parts which may proceed actively long before differentiation is complete.

The theory of differential periodicity maintains that the crystallographic pattern is propagated in the extension of the gene column, in which each lattice extends uniformly and comes into different degrees of alignment or stagger with the others. Subsequent studies have led the writer to extend the previous concept of superposed monolayers to a fully 3-dimensional system, in which the lattices are both tilted and rotated; the effect of the theory in its extended form is now more comprehensive, but the fundamental principles pointed out in the papers which are the subject of this review are the same. The result of the extension of the genic substance is to build up a differential periodic pattern, or moiré pattern. Hence we can test the theory by means of

moiré patterns. If it is right such patterns should resemble morphological patterns. That they actually do so is indisputable. The sheen of watered silk carries a pattern which is indistinguishable from the graining of wood. The same kind of pattern is also frequently produced on glazed paper, and the reader may be able to verify immediately what has been stated, for such paper is commonly used as the binding of exercise books. In other cases he will note patterns resembling cellular fields, mottlings and streak-like effects which recall various organic formations. An example is shown in Figure 10. The special research work which has been done in connection with the theory consists of the construction and close examination of differential periodic fields. These yield many general explanations of morphological peculiarity. Radial and bilateral symmetry are immediately explicable, to which may be added asymmetry. Annular formations are also of frequent occurrence in moiré fields as they are in the organism. The fundamental relationships of floral parts can be found in the simplest possible differential periodic figure, consisting of two inclined systems of circles, or other simple units, in which the whorling and alternation of elements occurs as in a flower. By using a larger number of components more complex floral schemes can be matched, sometimes with a lifelike effect. Resemblances to a number of pigmentation patterns have been produced also by two systems of circles. In these simple cases the interpretation is that the morphogenetic lattices, which will be actually numerous, interact so that the general effect can be integrated as the interaction of two components. Further unpublished studies have led to extensive matching of almost all types of morphological pattern, including the forms of insects with the appendages correctly shown. The discovery has thus been made that geometrical figures of a certain class have the form of organic pattern, and the figure of the organism has been elucidated. It is undoubted that all living pattern exists on a common basis and expresses a geometrical law of unsuspected simplicity.

Moreover these patterns throw light upon morphological phenomena which have been misunderstood and may have carried false implications. It cannot be denied that the idea of fortuity which plays so large a part in evolutionary theory, is derived in part from the impression of irregularity and haphazardness which one obtains from the immediate inspection of many if not most organisms, whose mottlings, variable serrations, etc., seem to prove the existence of some form of

inadequacy or chance in the basis of the organism. But exactly the same kind of apparent irregularity is found in differential periodic configurations which are constructed on the basis of extreme accuracy. Their existence in the organism is now intelligible, and the term irregular merely means that the geometry of the organism has not been understood.

A differential periodic pattern possesses the capacity for reorganization to give localized change, that is to say, by a slight rotational or translational shift of the components a portion of the pattern can be greatly modified, the effects diminishing as we proceed away from this point. This agrees with what is encountered in comparative anatomy, for frequently a species will show high differentiation of a small region of the body while the remainder may be closely similar to the ancestral condition. Moreover, this statement is a paraphrase of the law of heterogony, in which a slight change in size is accompanied by a pronounced alteration in the relative size of a part.

There appears to have been practically no previous work done in this field of geometry, for which the term differential periodic geometry is suggested. The writer has been able to discover only one paper on the subject (Lees, '19), which deals with the mathematics of a simple field of cross lines and is not accompanied by any suggestion that the subject may have a biological application.

Turning now to the most general properties of a differential periodic field, one notices: (*a*) The field is differentiated, (*b*) it is differentiated in a manner resembling organic pattern (Figures 2-9), (*c*) it has equipotential properties of regeneration and regulation.

The problem propounded by Driesch was: upon what basis can a differentiated structure exist so that a part of it can regenerate another part? There are certain natural and artificial systems in which a structure can impress or propagate its pattern, such as a printing machine or a crystal. But no known material system can be thought of having properties of pattern production corresponding to the organic, and thereby disproving the entelechy. This peculiarity is present in a differential periodic system, and the fundamental explanation is that in spite of its complexity the pattern is uniform in analysis. If a portion of the pattern is excised the remainder can regenerate it by plain extension of the lattices whose units again fall into the alignments determining the missing configuration. As regards regulation, it is seen that the different configurations in the pattern are due to different

degrees of translation of the lattices. Therefore any one can be transformed into any other by a translational shift, which may be a very minute movement. The maximum movement necessary is only half a period or lattice mesh.

Therefore the differential periodic system satisfies the requirements of a theoretical model of the organism in so far as regeneration and regulation are concerned. The limitations of these powers which are actually observed in most organisms present no difficulty, for when the theoretical system is incarnated as a chemical structure certain secondary phenomena will arise.

If a portion of the field is transplanted to another position it may be supposed to establish continuity with its surroundings, in which case the several lattices will connect with the same of the surrounding field. This process involves lattice translation, in which the theoretical possibilities are that either the implant or the host may suffer the translations. This is in agreement with facts relating to the organizer, for in some cases the implant organizes, in others it is transformed by the host. Organizing action is thus the crystallographic transmission of pattern. The extensive part which it plays in development is to be explained by supposing that certain degrees of alignment determine more stable interstitial groupings which tend to lock the lattices, so that when these alignments have been established they will control, or adjust if necessary, the pattern of the adjoining region of the field. In this process the cells influence one another by their protoplasmic connections, a cell being in equilibrium with its neighbor when the two gene columns have such an alignment pattern that they would fit if moved into contact. In this case the cytoplasm is postulated as an expansion of the interstitial or intergenic material of the chromosome. When the theory is extended to 3-dimensional components this postulation becomes unnecessary. At certain degrees of alignment the interstitial groupings represent relatively stable stereochemical configurations and produce an anchorage of the alignment pattern to cytoplasmic features, determining that certain regions of the embryo will dominate others. Hence the organizer appears to take control of one or another of the various developmental processes. There can be little doubt that the theoretical picture closely corresponds to the actual.

The utility of the theory is most apparent when we come to consider the form of the gradient field, for the geometrical fields can be readily plotted in great variety and the gradients in them can be seen in the

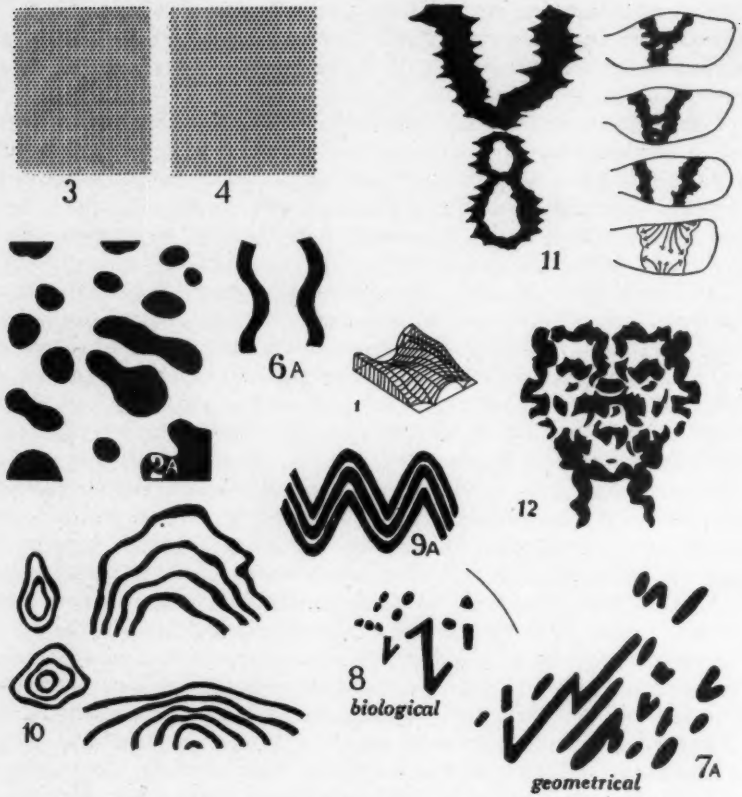

FIGURE 1

PORTION OF A WAVE FIELD IN WHICH GRADIENTS OCCUR IN VARIOUS DIRECTIONS AND ARE ALL DETERMINED BY THE SAME SET OF WAVE COMPONENTS

It is a gradient field of this form which, the theory maintains, must be considered as the basis of organic gradients.

FIGURE 2

DIFFERENTIAL PERIODIC FIELD, OR MOIRÉ PATTERN, PRODUCED BY SUPERPOSING FIVE SYSTEMS OF DOTS AT ANGLE INTERVALS OF 1°

A sample of the component is shown in Figure 3. The dark areas of the field are sketched in Figure 2*A*, and resemble a group of pigmentation mottlings, of various size, shape, orientation and spacing, as in the organism. The field must be viewed at an appropriate distance to obtain the required effect.

FIGURE 3

COMPONENT OF EVENLY SPACED DOTS

The angles at which the rows of dots are inclined are 61.8°, 59.2°, 59°.

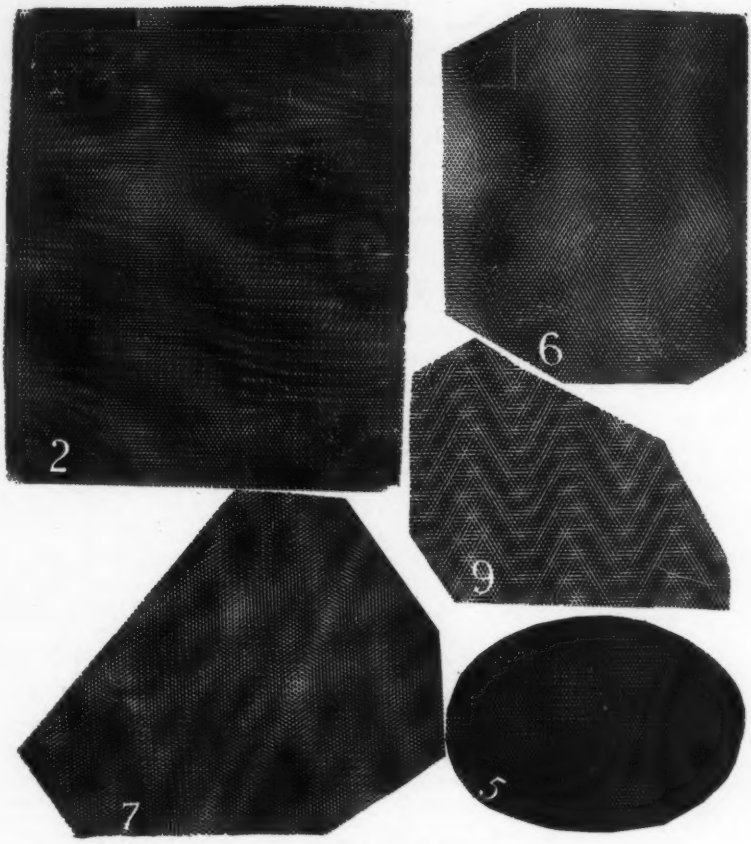

FIGURE 4

COMPONENT IN WHICH PERIOD IS LARGER
The angles are 58.2°, 58.2°, 63.6°.

FIGURE 5

FIELD FORMED OF SIX APPLICATIONS OF THE FIRST COMPONENT, AT ANGLE INTERVALS OF 1°
The dark areas form a coarse variable reticulum as in the pigmentation pattern of the horse.

FIGURE 6

PRODUCED BY THREE APPLICATIONS OF FIRST COMPONENT, AT ANGLES 0°, 1°, 30° TO BASE LINE
The dark areas, sketched in Figure 6*A*, depict the notochord in process of constriction by its sheath.

gradations from light to dark areas. The gradient pattern is then seen to exist in all directions (Figure 1). Its crests (Figures 1-9) may have the form of straight or curved ridges, peaks, craters, and crescentic elevations, variously arranged and orientated, yet the whole pattern exists upon the same uniform basis. It becomes unnecessary to suppose that one gradient exists for the body of an organism and another for a limb. The possibility that all gradients in the organism are really the same gradient completely eludes existing gradient theory in which the essential conception is lacking, namely that of a compound wave field. If we plot the gradient along a straight line, which might be the axis of the organism, we find that it may be long and gentle, or undulating and precipitous, corresponding to the gradual or sudden changes in body or limb form which occur in different types of animals.

Huxley's law of growth partition is exceedingly difficult to interpret, but on the basis of crystallographic control it becomes intelligible.

FIGURE 7

PRODUCED BY FOUR APPLICATIONS OF FIRST COMPONENT, AT ANGLES 0°, 60°, 61°, 62°, TO BASE LINE

The dark areas (Figure 7*A*) have the form of zigzags, *V*'s and small spots, as in Figure 8.

FIGURE 8

MOTTLINGS OF MOLLUSC, *Tapes pullastra*

FIGURE 9

PRODUCED BY THREE APPLICATIONS OF FIRST COMPONENT, AT ANGLES 0°, 1°, 60°, WITH TWO APPLICATIONS OF SECOND COMPONENT AT 0°, 1°

The dark areas have form of parallel zigzags, as in myotome pattern (Figure 9*A*).

FIGURE 10

MOIRÉ PATTERNS FROM EMBOSSED PAPER

The pattern is produced by the opposing pressures of countersunk rollers each bearing a uniform pattern of parallel slightly undulating ridges. The effect is to produce a wave pattern in the sheen of the paper, which is here traced in black. The pattern is practically identical with the scales of Salmonidae; *A* depicts a small scale with two circuli; *B*, with three; *C* and *D*, portions of larger scales. Note that circuli are of varying sinuosity, width, and spacing as in the real scale. All of these configurations are produced on the same uniform basis, showing that the young pattern is determined equally with the old on the same crystallographic basis.

FIGURE 11

MOIRÉ PATTERN FROM EMBOSSED PAPER CLOSELY RESEMBLING BANDS ON WING OF *Ephestia kuehniella*

The wing is shown, with one of the diagrams used by Goldschmidt, to explain the pattern in general terms, and on basis of complex sub-hypotheses, whereas theory of differential periodicity gives direct explanation.

FIGURE 12

HUMAN FACE PRODUCED BY DIFFERENTIAL PERIODICITY

The figure is produced by stencilling on the basis of differential periodicity. The boundaries are arcs of eight systems of regularly spaced circles, selected in a consistent manner. The figure depicts locks, moustache, beard, etc. Eyes are low as in child, gibbon, and probably ancestral man. At a distance it assumes realistic aspect of face of savage.

The theory of differential periodicity maintains that a differential periodic pattern is maintained at all stages of development, which differ in respect of the alignment pattern. The extensive evidence collected by Goldschmidt for his theory of process rates is immediately available as support for the present theory, for Goldschmidt's arguments can be readily transcribed into rhythmic rates and for these we can substitute a spatial instead of a time factor. The time factor is merely an accompaniment to the spatial. The wave field concept interprets the genetic facts brought together by Goldschmidt under the term phenocopy. In these cases a character which is absent if the genes act under normal conditions can be brought into existence by various experimental agencies (abnormal temperature, narcotics, CO_2, etc.) just as though the organism contained the gene for this character. Now, since the characters of the organism are determined not by individual genes but by the molecular rhythm of the genes arising from their inclinations, the differential wave components can be altered in length or translated so that a crest in the gradient field can be moved, reduced, or accentuated. The crest may move outside the organism so that it is now virtual, and the organism appears to lack a certain character. But, nevertheless, it is potential for this character and it may be a simple matter to shift it back into the position of reality. This may be effected by any agency which distorts the morphogenetic lattices; hence it comes about that so many different agencies have the same effect, and that experimental disturbances of morphogenesis rarely produce characters which are new. Hence Goldschmidt has been led to his idea of a "floating gene," and further to the view that the abandonment of the gene concept may be necessary and even overdue. All of this is made clear by the theory of differential periodicity. Existing cytogenetic theory has the defect that it considers only one of the theoretical possibilities in regard to the spatial relationships of the gene, namely, its position on the gene column. It takes no account of the orientation of the gene. The gene column cannot, however, be compared to a string of beads in which the effect is the same however the beads are turned.

One of the outstanding difficulties of biology does not arise in the theory of differential periodicity. This has already been referred to, namely, the problem as to the extent to which the basis of heredity is to be apportioned between nucleus and cytoplasm. The theory represents the cell as an integral whole, in which the structure of the cytoplasm is related to that of the nucleus as the parts of a machine are

related to their controlling levers, and in which a lever may move a part or a part may transmit movement back to a lever. The fact that levers and parts are accorded unequal status may have little significance in either case. The part which we choose to represent the basis of heredity is that which is most easily seen, namely, the chromosomes. But the pattern in which the machine exists, and by which it can produce effects, can be equally well stated in terms of levers or parts. The alignments of the genes are expressible as the nature of their interstitial connections, which are directly or indirectly the cytoplasm. It is frequently assumed that three sources of hereditary characters exist, namely, the male genes, the female genes, and the maternal cytoplasm. But these, though they may be physically distinct are in fertilization welded into one resultant pattern, which involves translations or rotations in the lattices accompanied probably by stresses. Sometimes a part will yield to another; sometimes it will dominate.

The effect of these stresses is to produce tautness in the diploid system, and its superiority to the haploid is that of a taut instrument to a flaccid. Further studies, however, have led to the conclusion that the essential reason for diploidity must be stated more completely than this. The fundamental significance of it is in the reinforcement of two wave fields, which, however, must not be identical or they will undergo coalescence into one by lattice translation. Therefore self fertilization is of little or no advantage and inbred strains weaken because their genetic differences are insufficient to supply this factor. The strained double system, after serving the organism well through its development and life, by determining high synthesis, sensitivity, and response, reduces its strain by the interchange of portions in crossing-over. If two threads are twisted in different patterns it is possible to exchange portions so that the threads will pair more easily. This is the essential nature of crossing-over. But now that the threads have been made more closely similar their morphological potencies have also been made more closely similar. The effect of crossing over is to reduce morphological differences within the species. This is a positive effect and different from what is supposed in cytogenetics, where the mere random flow of genes must be regarded as the cause of specific uniformity. Great dissimilarity between the parents may lead to the failure of crossing-over because, in order to produce the exchange, portions of the gene columns must be capable of uniting and forcing other portions to cross over. If the former are insufficient the process will fail to take place,

and the species will divide, for the unreduced dissimilarities are free to proceed to the point when the patterns will be too different to fuse at fertilization, so that divergence is coeval with infertility. Cytogenetics cannot integrate specificity and sexual potency by any fundamental explanation; the theory of differential periodicity can do so.

The theory also predicts a lower limit of crossing-over, for when dissimilarity is slight there is less need for crossing over. Thus Mather ('36) states that inbred rye has low chiasma frequency. It will be seen, further, that the above conclusions, which are arrived at from geometry, are identical with those of Darlington who considers that crossing-over serves to reduce the strain of interrelational coiling, but can suggest no relation of such a fact to morphology.

Turning now to matters of chemistry, one observes that the extensive and variable superpositions, interlockings, or interpenetrations, postulated by the theory may be related to the large open meshes and extensive internal surfacing which has been established for colloidal formations. The theory introduces an idea into megachemistry which, in spite of its obviousness, appears to have been overlooked. It is almost invariably assumed by protein chemists that molecular structure must exist upon a basis of simple commensurability, i.e., the groupings must fit conveniently. Thus molecular diagrams, including those constructed to illustrate theories of chromosome structure [Wrinch ('36); Gulick ('39)], are drawn with their units superposed in regular tiers after the fashion of a scaffold in which beams are arranged in simple parallel and perpendicular formation. It is clear that this assumption merely expresses human ideas of neatness and convenience. The "geometrically permissible" of Wrinch, the "appropriate spacing" of Schulman and Rideal ('37), the "effective" and "successful" superposition of Gulick, illustrate clearly this tendency of thought. But we have no assurance that nature operates according to human ideas. It is no law of nature that the incommensurable is the impossible. If lattices are superposed so that units do not correspond, and vary infinitesimally in their degrees of alignment, it cannot be assumed that combination is impossible. They may combine, for example, in a state of strain, in which units or residues obstruct, or are connected by valencies tilted from their normal angles. The theory of chemical strain, though not in a biological connection, is well known (Baeyer, '85), and the increased chemical activity which it predicts is now available as the basis for the high synthetic activity of protoplasm. The low temperature oxidations of

fats, sugars, etc., which have remained largely enigmatical, receive an interpretation if we assume that the strained pattern of the protoplasm, either directly or by way of an enzyme, is imparted to them, or to oxygen, by temporary union with a strained moiety in the enzyme.

The spirit of the theory, as will be seen, is that the mathematical basis of differentiation is the key to biological properties in general, which are all corollaries one of another. It extends equally to the broadest biological matters, such as adaptation. The theory of fortuitous variation is rejected on the grounds that the probability is too low for concatenations of adaptive characters so extensive as those of the organism to have come about by chance. The theory of differential periodicity, in the first place, offers a new theoretical basis for the inheritance of acquired characters. It supposes that the germ plasm can be subjected to organizing action proceeding from a modified portion of the soma. There is no reason to suppose that organizing action, which plays so large a part in development, is entirely lost in the adult. Even if most of the definitive structures can resist the organizing action of a modified part, channels may exist whereby the action, which is expressible as propagated lattice shift, can reach the germ plasm. Any change in the organism, local or otherwise, which is of a non-disruptive nature, necessarily involves lattice shift. It is further supposed that the nerve impulse is lattice shift propagated at high velocity; the theory thus aligns itself with the school of thought favouring the chemical as opposed to the electrical theory of nerve transmission. Since extirpations do not theoretically reorganize a differential periodic system, the non-inheritance of such effects is consistent with the theory.

However, the theory does not derive adaptation solely from the inheritance of acquired characters. It refers in particular to the peculiar nature of differential periodic pattern in which the basis of coördination is inherent. The coördination of parts is the principal factor in adaptation. Since the pattern is expressible as a wave field, the crest of the wave can be considered to have antithetical properties to the trough. The pattern can be regarded as consisting of a complex system of interlocked antitheses in which structures highly specialized in one direction will be accompanied by those specialized in another, or, what is equivalent, degenerate. It is to be supposed that the geometry of the environment has necessarily something in common with the geometry of the organism, and that the final explanation of adaptation will depend upon a fuller visualization of differential periodic properties. Since both

environment and organism are derived from the same substratum of existence a relationship may be inevitable between them, and the problem of adaptation may be obscured by the error of regarding organism and environment as though they belonged to two different systems of existence. The only change which is possible in a differential periodic system is the transformation of a wave crest into its antithesis, the trough. If the first is unadaptive, the antithesis will presumably represent the adaptive condition, so that the mechanism automatically adjusts itself to specific environment. It may be possible to transcribe this into concrete terms in regard to chemical adaptation, as seen in immunity, for the shift of the lattices may bring into existence groupings which can combine with or otherwise render harmless the toxic substance. As far as structural adaptation goes, the complex of factors is so difficult to envisage that we may have to be satisfied with an abstract or semantic explanation. If so, adaptation will never be directly understood, but its existence will be understandable.

The theory also maintains that sexuality is involved in adaptation. A given environmental effect acts differentially upon the two sexes, the resulting modifications being integrated in the interactions of germ plasms into an effect which represents not mere correction or corroboration or amplification, but brings out some added and important aspect of the environment, on the analogy of stereoscopic vision. Further studies have led to the view that the sexes are separate configurations in the same differential periodic field, which theoretically extends to infinity.

The origin of the organism took the form of the building of a differential periodic system. In an ordinary crystal there exists rhythm, but not interplay of different rhythms. The crystal is therefore undifferentiated, but if more than one rhythm can be introduced it is now a differentiated harmonic equipotential system. Driesch's concept was sound, and in introducing this term he made the most fundamental of all generalizations regarding the organism. But he failed to discover the theoretical basis for such a system and it is to be assumed that he looked for complexity where simplicity actually existed. Ordinary mineral substances are not adapted for the building of such a system, since their crystalline structure is too fine and close to allow interpenetration of one crystal by another of different period, or relatively inclined. Therefore life originated in the form of matter whose structure permitted of differential periodicity. There are several theoretical

possibilities in this connection. The simplest is that a protein lamella in the form of a shell enveloped another one, so that the different curvatures determined the difference in period; such a system is not limited to two layers. Such a formation has actually been suggested for the structure of the virus particle by Barnard ('32) who assigns to it a coccoid form with an internal cavity containing water on the basis of ultraviolet photographs. The second possibility is that flat protein lamellae became united in a state of inclination, like a pile of mats variously crossed. The third is that space lattices became interpenetrated. In such systems the effect may be to produce the appearance of homogeneity, since in one respect the system is uniform, namely in that the same lattices exist in all parts. Bawden and Pirie ('37) compare the difference between the virus and a crystal to that between a loose pile of matches and the bricks in a wall. Now in a differential periodic pattern the crisscrossing of lattices actually gives an effect which may be indistinguishable from a random effect. This is quite obvious if a number of grids are ruled on a piece of paper at different angles. The pattern may appear quite chaotic and in any event it is almost impossible to say how it has been produced except by a conjecture. The last quoted workers refer to the virus solution as a 3-dimensional mosaic of regions arranged at random but in each of which the molecules lie parallel. Various other workers have described orientated systems and have postulated the sliding of groupings. Therefore much evidence already exists which could be used to support the theory of differential periodicity, for those who might be unwilling to consider it except upon the basis of direct chemical fact. At the same time the theory is not tied to any specific chemical postulations. Darlington believes that the structure of water plays an important part in chromosome mechanics, and it is conceivable that the determining lattices which are the basis of the theory might be water, in which case the proteins represent the interstitial materials. On this basis the origin of life is to be found in an acquired structurization of water, whose possibilities in this respect are yet very imperfectly known. As against this possibility, Bernal and Fankuchen ('37) state that the virus contains no internal water. Whatever the essential ground substance is, the course of evolution is represented by alterations in the angulation pattern in which the lattices are associated, the effect being to transform the pattern of the differential periodic field, and hence the form of the organism.

Life, then, is a very much simpler thing than has been suspected. It is not a special or accidental phenomenon or one whose explanation involves references to principles or properties beyond the range of ordinary human understanding. It is the most fundamental aspect of material and mechanistic existence as will be realized if the definition which has been given here of life is considered, for the figure so specified is the most general and unspecialized of all forms of organization. It is the capacity for variation of such a figure which has given rise to the belief that life is infinitely complex, delicate and unstable. It is actually highly stable, and the reason is that simplicity is at the basis of its organization. Scientific understanding has advanced relatively rapidly in regard to other branches of natural phenomena because it has been preceded by the development of the appropriate branches of mathematics. But one branch of mathematics has not undergone development, and this is differential periodic geometry. This hiatus in mathematics is related to the greatest hiatus in human understanding, namely that which exists in regard to the nature of life.

Summary

Organic form expresses a pattern which can be simulated geometrically by interpenetrated systems of evenly spaced points. The figure of the organism has been elucidated: it is a moiré pattern in solid geometry. The mathematical basis of differentiation is differential spacing. The organism is a crystallographic pattern determined by the interplay of molecular rhythm arising in the interaction of inclined lattice systems. It is subject at all stages of its development to crystallographic control of form, and the facts of heterogony, growth gradients, genic rate processes, are related to this but the basis has remained unsuspected. Organizing action is the crystallographic transmission of pattern, involving shifts or rotations of the component lattices, which are specifically represented in the structure of the gene column, to which the structure of the cytoplasm is directly related. The process is represented, at an extreme of slowness, in the modification of the germ plasm by somatic change; at an extreme of rapidity, in the transmission of the nerve impulse.

A differential periodic field contains a wave pattern in which various components interact. Therefore the properties of wave systems can be used in general for the interpretation of biological properties. The absence of a genetic character implies the shifting of a wave crest to a

position outside the organism, the character being now virtual and the organism still potential for it. Hence experimental disturbance of morphogenesis by temperature, etc., produces characters which are not new but found normally in other individuals.

The periodicities of the components are in general incommensurable, permitting no connection by valencies in regular formation, or, alternatively obstruction of units, leading to chemical strain which is the basis of the synthetic and other activities of protoplasm. Diploidity involves the reinforcement of two wave fields, which must be slightly different to avoid geometrical coalescence; they are fused in a state of strain, adding to the strain of the haploid system. At crossing-over the strain is reduced by the interchange of portions. The significance of crossing-over is a geometrical one. From the relation of the pattern of the chromosome to the general morphological pattern, crossing-over controls somatic dissimilarity within the species, integrating specificity and capacity for fertilization on a fundamental basis.

Various properties of fundamental adaptive significance are inherent in a differential periodic system and not the result of selection, namely, high synthesis, power of regeneration and regulation, high dissemination through minuteness of minimum effective reproductive fragment, capacity for reorganization to give localized change and hence permit evolutionary advance without sacrifice of previous pattern in respect of other parts.

The system is differentiated into antithetical properties; change which is non-disruptive necessarily replaces a property by its antithesis or varies it between the limits of an antithesis, explaining somatic adaptive change, for the equilibrium condition is necessarily reached.

The organism originated when matter became organized on the basis of differential periodicity. Life is definable as the expression by matter and energy of the properties of a figure consisting of systems of evenly spaced points. Nothing appears to have been known of such figures and their properties.

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EFFECT OF l-PROLINE ON DIFFERENTIATION IN ULVA*

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A. INTRODUCTION

In the past this laboratory has shown histologically and anatomically that l-proline stimulates cell differentiation in a large variety of animals, including cancerous mouse tumors (Hammett and Collings, 1937; Hammett, 1940; 1941; Hammett, Hammett, and Goldsmith, 1940; Wilson, 1941).

Attempts to extend this finding to plants, using root-tips of seedlings, gave no data from which conclusion could be made (Barghoorn, 1942). It was therefore decided to try marine algae.

This paper will be confined to our experiments with the green alga *Ulva*. This plant, in spite of its uniform cellular structure presents suggestive criteria for the determination of cellular differentiation.

B. MATERIALS AND METHODS

In choosing materials for the general study the following species of algae were examined because of their flattened blades: *Fucus sps.*, *Chondrus crispus*, *Enteromorpha sps.*, *Laminaria sp.*, *Porphyra atropurpurea*, and *Ulva lactuca*.

All the above materials, when collected in good condition, show a cellular increase when grown in fresh and frequently changed sea water under laboratory conditions.

The experimental methods described herein make available a number of algae which hitherto have not been used to any great extent for growth and physiological experiments.

Of the forms mentioned *Ulva* was chosen for the following reasons: It is easily obtained since only *Fucus* exceeds it in abundance along the upper Cape. It has the advantage that it is only two cells thick. It has no auxiliary pigments which block microscopic vision when studied *in vitro*.

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While *Enteromorpha* and *Porphyra* are but one cell thick, the former curls when cut into discs, and the latter is rubbery and difficult to cut without distortion. There are doubtless other marine algae which could be used.

Fresh material was collected at the Provincetown Breakwater. Care was taken to collect the *Ulva* while submersed and to take it back to the laboratory in fresh sea water, not only to keep it fresh, but also to prevent fruiting.

The experiment was set up shortly after collection. First, small culture dishes were set up with 30 cc. of fresh sea water in each. Then discs were cut out of the fronds by placing the leaf over a large cork and cutting with a sharp 5 mm. cork-borer. It was found that if the cork-borer was kept sharp and precaution taken against stretching of the leaf, the discs could be uniformly and cleanly cut.

The discs were cut from the greener and slower growing region an inch or two above the holdfast end of the leaf, because it was found that during reproduction, the marginal and tip end cells often fruited soon after cutting.

It was found that experiments set up earlier in the season gave better results than those set up in August, partially due to the vitality of the material, and partially to environmental factors.

Discs cut close to the holdfast should not be used because such usually fail to grow.

Fifteen discs were placed in each dish to allow for loss due to death, solution changes, and fruiting. Only the ten largest discs were used for measurements. Those in which no appreciable growth took place were discarded in both tests and controls.

Two experiments were run with the following concentrations of l-proline, viz.: M/200,000, M/100,000, M/50,000, M/20,000, and M/10,000. One experiment contained M/5000 l-proline.

Solutions were changed three times a day at early morning, noon, and late afternoon or evening, about five hours apart. Only fresh filtered sea water was used.

Test and control cultures were run simultaneously under like conditions of pH, temperature, and illumination.

Since the M/50,000 and M/20,000 concentrations seemed the optimal two other series of experiments were run thereat as checks using larger numbers of discs, viz.: 50 and 30 respectively.

Experiments were run from five to seven days. The gross diameter

of the discs was measured after the period of growth in microns, using a Bausch and Lomb ocular micrometer with 6x ocular and 32 mm. objective. The number of marginal cells along five contiguous arcs 200 micra long was counted, using a Leitz net micrometer with 10x ocular and 44x objective.

From these two measurements the circumference was computed, as well as the approximate number of cells therein, and the average width of the marginal cells.

It was early noted that the discs in the l-proline cultures were darker green than their controls. Since it was not possible to count the number of chloroplasts an attempt was made to estimate the difference in chlorophyll concentration colorimetrically.

In this experiment the largest cork-borer was used to cut out the discs. At the end of eight days in M/20,000 l-proline each disc was recut with the same size cork-borer so that both test and control discs would have the same area. An attempt was made to compare the greenest test discs with the greenest controls. An alcohol extract was made of five discs from each and the chlorophyll difference measured in a Klett colorimeter.

C. Results

In Table 1 are the results of the effect of the various molar concentrations of l-proline upon the size of *Ulva* discs, as well as the average width of the peripheral cells of the discs. These results are visualized in the accompanying graph (Figure 1). The graph pictures the percentage difference of test values from control at the several concentrations of l-proline. The data can be summed up as follows:

1. l-proline induces a general increase in the diameter of the disc, the number of cells in the circumference, and the diameter of the peripheral cells of the disc.

2. The effect is roughly proportional to the concentration; being least at the lowest value, rising as the amount of l-proline is increased to the apparent maximum at M/20,000, and diminishing with further increase as toxicity begins to show an influence.

The data of Table 2 substantiate the effect at the critical concentrations. The difference in degree of response may be due either to difference in state of the *Ulva,* since these experiments were run in August; or to a temperature difference; or perhaps to the fact that the larger number of discs used provided more reliable results.

TABLE 1

THE EFFECT OF l-PROLINE ON THE GROWTH OF *Ulva* DISCS OF 5 MM. DIAMETER

	Control	M 200,000	M 100,000	M 50,000	M 20,000	M 10,000	M 5,000
No. discs (Total)	20	20	20	20	20	20	20
Diam. mm.							
Expt. 1	5.07	5.43	5.45	6.18	6.38	5.68	5.52
Expt. 2	5.20	5.36	5.44	5.72	5.80	5.62	——
Average	5.14	5.39	5.44	5.95	6.09	5.65	5.52
% Incr. avs.	——	*4.9*	*5.8*	*15.7*	*18.5*	*9.9*	*7.4*
Circum. mm.							
Expt. 1	16.93	17.06	17.12	19.42	20.04	17.84	17.34
Expt. 2	16.34	16.84	17.09	17.87	18.22	17.66	——
Average	16.63	16.95	17.10	18.64	19.13	17.75	17.34
No. Cells .02 mm.							
Expt. 1	11.6	11.2	11.6	10.7	10.5	11.0	13.4
Expt. 2	14.4	15.0	13.8	13.5	13.3	13.6	——
Average	13.0	13.1	12.7	12.1	11.9	12.3	13.4
% Incr. avs.	——	*0.8*	*—2.3*	*—6.9*	*—8.5*	*—5.4*	*3.1*
Cell Width mu							
Expt. 1	17.2	17.9	17.2	18.7	19.0	18.2	14.9
Expt. 2	13.9	13.3	14.5	14.8	15.0	14.7	——
Average	15.5	15.6	15.8	16.7	17.0	16.4	14.9
% Incr. avs.	——	*0.6*	*1.9*	*7.7*	*9.7*	*5.8*	*—3.9*
No. Cells Circum.							
Expt. 1	981.9	955.5	993.0	1040.0	1052.0	981.2	1161.8
Expt. 2	1176.5	1263.0	1179.2	1206.2	1211.6	1200.9	——
Average	1079.2	1109.2	1086.1	1123.1	1131.8	1091.0	1161.8
% Incr. avs.	——	*2.8*	*0.6*	*4.1*	*4.8*	*1.1*	*7.7*

A third experiment was set up, but swarm spores developed on the fourth day, making it impossible to complete the trial in the accustomed manner. It was noted, however, that the layer of marginal cells which were not swarming was thicker in the tests than in the controls. So counts were made of the number of cells comprising this thickness. These are given in Table 3. Again the maximum increase is at M/20,000, a nice confirmation of the previous results.

As previously stated, in all of the experiments, one was immediately struck with the difference between the greenness of the discs in the test and control cultures. When the greenest discs of the two sets were compared it was found that the tests contained two per cent more chlorophyll. In the paler members of the series, however, the concen-

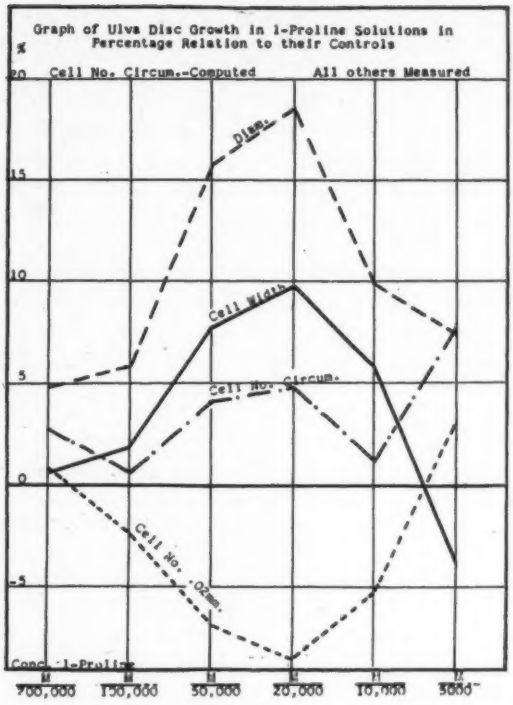

FIGURE 1

tration of the tests increased to nearly 12 per cent over that of the controls. The average increase in the l-proline cultures was 6.6 per cent.

Finally it was noted that the cell walls of the marginal cells grown in l-proline cultures were quite generally thicker than those of their comparative controls.

D. Discussion

Although the findings with animals give a more striking picture of the effects of l-proline on differentiation, *Ulva* does present evidence that a simple multi-cellular plant tends to react similarly.

Increase in diameter of the discs grown in l-proline was first thought to be possibly due to increase in cell number or proliferation. After counts had been made of the number of marginal cells in unit arcs,

TABLE 2

THE EFFECT OF M/50,000 AND M/20,000 l-PROLINE ON GROWTH OF *Ulva* DISCS OF 5 MM. INITIAL DIAMETER

	M/50,000		M/20,000	
	Cont.	Test	Cont.	Test
Number discs	50	50	30	30
Days growth	6	6	7	7
Diameter mm.	7.39	8.24	6.89	7.30
% Incr. tests		*11.5*		*6.1*
Circum. mm.	23.22	25.89	21.65	22.97
No. Cells .02 mm.	11.8	10.7	12.5	11.9
% Decrease tests		*—9.3*		*—4.8*
Cell Width in mu	17.0	18.7	16.0	16.8
% Incr. tests		*10.0*		*5.0*
No. Cells Circum.	137.0	138.5	135.3	136.7
% Incr. tests		*1.1*		*1.0*

TABLE 3

AVERAGE NUMBER OF VEGETATIVE CELLS IN CROSS-SECTION OF MARGINAL LAYER OF SWARM SPORE PRODUCING *Ulva* DISCS

Concentration	No. active discs	No. cells in layer	% incr. tests
Control	4	2.55	——
M/200,000	3	3.13	22.7
M/100,000	3	3.87	51.8
M/50,000	4	5.60	119.6
M/20,000	5	6.20	143.1
M/10,000	4	5.75	125.5
M/5000	3	5.79	127.1

however, it was found that these were pretty generally less in number in the tests, and that the total number of cells in the greater circumference was not so very much greater than in the controls. At least not enough to account for the difference in diameter as the measured value.

This was particularly true in the supplementary experiments with 50 and 30 discs subjected to M/50,000 and M/20,000 respectively. Here the increase in cell number in the circumference of the test over that of the control was essentially negligible being but a bare one per cent.

The measurements of the width of the marginal cells revealed that

these were consistently and generally greater in the discs exposed to l-proline than in the control discs.

This greater increase in cell size as compared with the increase in cell number is suggestive that the cells were stimulated to maturity somewhat more than they were stimulated to proliferate. Compare the line for cell width with that for cell number in the chart.

And since the cell walls of these large cells were thicker than those of the smaller control cells, the validity of this deduction seems established.

In the experiment in which swarm spores developed shortly after the experiment had been set up, there was a definite correlation between the l-proline concentration and the average number of cells in the marginal cell layer thickness. The results are consistent with those obtained with respect to cell size increase. Further there were more vegetative cells at M/20,000 in this out layer than at any other concentration of l-proline, and the effect at all concentrations was definite.

Since the shift in growth activity which occurs when a vegetative cell becomes a swarm spore cell is as yet unknown, the interpretation of the restraining effect of l-proline thereon must be uncertain.

It could be that l-proline acts here as a retardant of proliferation as it did in *Obelia* (Hammett and Collings, 1937). Or it could be as suggested by the fact of cell size and cell wall increase, that the *Ulva* cells can be stimulated to a slightly greater maturity than they customarily attain; that this maturity puts them beyond the stage where they can revert to swarm spore cells; and hence that the inhibition of cells in l-proline culture to go on to form swarm spores is further evidence of differentiation forwarding by this amino acid.

It might be noted here that increase in thickness of cell wall is as much a mark of a specialization process as is increase in thickness of septal chitin in the regenerating hermit crab chelae and that it is similarly interpretable (Hammett, Hammett, and Goldsmith, 1940).

There are several references in the literature to the effect of proline upon the nutrition of animals and blood. But very little if any work has been done with proline and chlorophyll of plants. Sure (1924) was one of the earliest workers to show that proline is one of the indispensable amino acids for growth, and Kapfhammer (1930) found it essential to nutrition. A year later two papers appeared (Galamini, 1931; Drabkin and Miller, 1931) which showed that proline was effective in the regeneration of red blood cells (hemoglobin) in rats, thus

relieving anemia. In view of this finding it is not surprising that this particular amino acid has a similar effect upon chlorophyll since this compound is chemically allied to hemoglobin.

In view of the fact that chlorophyll production is a specialized process; and since cell specialization is a mark of differentiation; it follows that the increase in chlorophyll production attributable to l-proline in these experiments is further evidence consistent with the idea that cell specialization or differentiation is forwarded by this amino acid.

There have been no other experiments which demonstrate the ability of l-proline to forward differentiation in the plant kingdom. Farber (1938) produced evidence that the compound increases the number of cells of the bacterium *Staphylococcus aureus,* but no attempt was made nor could any be, to show that there was any differentiation effect. Traub (1938), in testing some 100 compounds for root formation, found that l-proline was one of the best for root response in *Passiflora quadrangularis* and *Begnonia venustic.* The particular growth activity involved, however, was not clarified. Barghoorn (1942) also reported that M/100,000 l-proline stimulated root growth in length as measured from apex to protoxylem using *Phaseolus vulgaris and Gossypium hirsutum.* Neither of these root tip experiments, however, has given any satisfactory evidence that l-proline affects differentiation. Nor could they, since the experimental procedure was inadequate for this purpose.

The work on *Ulva,* however, does demonstrate beyond reasonable doubt that this amino acid does act to forward differentiation in plant tissue as it does in animal.

E. Summary

Discs of the green marine alga *Ulva lactuca* were allowed to grow in fresh sea water cultures containing l-proline in concentrations ranging from M/200,000 to M/5000.

There was a consistently greater increase in size of disc and size of marginal cells in the l-proline solutions with the maximum reaction obtaining at M/50,000 or M/20,000.

The cells of the l-proline cultures built thicker walls and produced more chlorophyll than the controls.

There was retardation of shift from vegetatives to swarm spore cell.

These data are taken as indicating that l-proline may act to forward cell specialization processes or differentiation in plant material.

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THE OSMOTIC VS. THE RESPIRATORY RÔLE OF SUCROSE IN THE NUTRITION OF EXCISED TOMATO ROOTS

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No matter how simple a particular substance may be, its rôle in the nutrition of any plant or animal is seldom an uncomplicated one. It is therefore important in any nutritional study to determine for each substance how many separate rôles can be distinguished, which of these rôles are indispensable, in which indispensable rôles the particular substance is replaceable by other substances, and what are the optimal conditions for the fulfillment of rôles which are both indispensable and irreplaceable.

Sucrose is no exception. Sucrose obviously fulfills at least two major rôles in the economy of excised tomato roots. In the first place it is the major source of respiratory energy, the major combustible material available to the tissues. This is an indispensable rôle. Sucrose appears to be essentially irreplaceable in this rôle since substitution for sucrose of equimolecular concentrations of any of a number of other sugars gives inferior results (White, 1934, 1940*a*, *b*; Bonner, 1940; Bonner and Addicott, 1937. See, however, Robbins and Bartley, 1937; Robbins and Schmidt, 1938). Nor does sucrose contain significant amounts of non-carbohydrate nutrient elements (White, 1940*b*). Its purely nutritional rôle is thus a relatively simple one.

Sucrose does, however, perform a second major function in all plant tissue nutrients, as an osmotic agent. In animal tissue culture nutrients an isotonic value is considered essential. It is usually obtained largely through the use of sodium chloride. Thus in Tyrode's solution (Parker's formula, Parker, 1938, p. 36) out of a total osmotic value of 4.37 atm., 3.34 atm. (76%) are supplied by NaCl, 0.9 atm. by salts other than NaCl (chiefly $NaHCO_3$), and only 0.13 atm. (3%) by carbohydrate, in this case, glucose. In this laboratory's standard nutrient, on the other hand, with a total osmotic value of 1.58 atm., there is no NaCl or $NaHCO_3$, the total salt content provides an osmotic value of 0.17 atm. (11% of the total), and the carbohydrate (sucrose) provides the remaining 89 per cent (1.41 atm.). This marked difference be-

tween the basic nutrients for animal and plant tissues makes highly desirable a careful analysis of the osmotic rôle of carbohydrate in the two nutrients and an estimation of the position, if any, of NaCl as a possible substitute for carbohydrate in this rôle in plant nutrients. That NaCl is not itself indispensable has already been clearly established. The work presented here was planned to investigate this question, as regards the nutrition of excised tomato roots.

For this purpose cultures were carried out using nutrients in which, at each of a graded series of total osmotic values, sucrose was partially or wholly replaced by NaCl so as to provide a simultaneous graded series of sucrose-to-NaCl ratios. An osmotic value of 1.5 atm. is known to be approximately optimal in the absence of major osmotic agents other than sucrose. A series of solutions was arranged to provide total osmotic values, exclusive of the small value due to nutrient salts, of 0.1, 0.3, 1.0, 3.0, and 10.0 times this value. Sucrose-to-NaCl ratios, prepared on a mol-equivalent basis of 1:0, 100:1, 30:1, 10:1, 3:1, 1:1, 1:3, 1:10, 1:30, 1:100, and 0:1 were tested at each of these osmotic values. An additional series was carried out in which NaCl concentrations to provide osmotic values of 0.15, 0.45, 1.5, 4.5, and 15 atm. were superimposed on a constant sucrose value of 1.5 atm. This made a total of 60 nutrient combinations. Twenty roots were grown in each solution and were maintained through two consecutive one-week passages, making a total of 2400 roots. The roots were from our standard clone, which was in the 488th *in vitro* passage at the beginning of the experiments, and were carefully selected to provide as uniform material as possible. The basic nutrient has been described elsewhere (White, 1942, 1943) as have the general laboratory procedures. Cultures were maintained in diffuse light, at a laboratory temperature of 22-25° C. Cultures maintained in a complete nutrient containing 60 mM sucrose but without NaCl were used as controls. Results were recorded in terms of linear increments, reduced to percentage of the simultaneous control values. Data from these experiments are presented in Table 1.

From the data presented in the table it is clear that the optimal osmotic value was about 1.5 atm. This was true not only when this value was obtained with sucrose alone (uppermost horizontal column) but also in all solutions capable of supporting normal or nearly normal growth in which this value was obtained by combining sucrose and sodium chloride in various proportions.

Sodium chloride was tolerated without serious depression of root

TABLE 1

EFFECTS OF TOTAL OSMOTIC VALUE, SUCROSE CONCENTRATION AND MOL-EQUIVALENT RATIO OF CONCENTRATIONS OF SUCROSE AND SODIUM CHLORIDE ON INCREMENT OF EXCISED TOMATO ROOTS

No.	Total osmotic value in atm. / Mol-equivalent ratio of sucrose to NaCl	0.15	0.45	1.5	4.5	15.0	Optimum sucrose concentration in millimols
		Increment during the second one-week passage, expressed as percentage of the increment during that passage in a solution* containing no NaCl and 60 mM (ca. 1.5 atm.) sucrose					
1	1:0	2	26	*100*†*	21	4	60**
2	100:1	6	20	*87*	12	2	59**
3	30:1	1	5	*82*	26	4	58
4	10:1	6	8	*82*	33	11	55
5	3:1	1	8	*37*	*23*	1	40-120**
6	1:1	8	5	*15*	*14*	1	30-90
7	1:3	1	5	21	*44*	1	60
8	1:10	1	1	7	*14*	1	18**
9	1:30	1	1	2	8	*25*	20**
10	1:100	1	1	1	4	1	—
11	0:1	1	1	1	1	1	—
12	0.6 N sucrose plus NaCl to make, atm.	1.65	1.95	3.0	6.0	16.5	
	Increment, in % of control	*74*	70	38	33	1	66

*Control.

†Figures representing the optimal values at each sucrose-to-NaCl ratio are in italics. In the fifth (3:1) and sixth (1:1) lines the values at 1.5 and 4.5 atm. do not differ significantly, hence both are italicized. The shift of this optimum from the 1.5 atm. column first to both that and the 4.5 atm. colum, then to the 4.5 atm. column alone and finally to the 15 atm. column is worth emphasizing.

**The optimum in the first line was at 1.5 atm. obtained with sucrose plus nutrient salts but without NaCl. Actually only 1.41 atm. or 60 mM were provided by sucrose. In the second line 1/100th of this was provided by NaCl leaving $.99 \times 60 = 59.4$ mM sucrose. In line 5 two-thirds of the total was provided by sucrose and one-third by NaCl but the values at 1.5 atm. (60 mM) and at 4.5 atm. (180 mM) were not significantly different. Hence the optimal sucrose value must be set down as lying between $2/3 \times 60 = 40$ mM and $2/3 \times 180 = 120$ mM. In the eighth line $1/10 \times 180 = 18$ mM, and in the ninth line $1/30 \times 600 = 20$ mM.

growth so long as its concentration did not exceed 6 mM. It was, however, somewhat depressant in all combinations and definitely injurious whenever its concentration exceeded this value, that is, whenever its concentration exceeded one-tenth that of the other osmotically active substances. This maximum (6 mM) is about three times the concentration of any other salt used in the basic nutrient.

While 1.5 atm. was the only osmotic value which gave fully satisfactory results, this does not mean that it gave optimal results under

all conditions. When the sucrose: NaCl ratio was 3:1 or when equimolecular concentrations of the two substances were used, the results at 1.5 atm. were not significantly superior to those at 4.5 atm. When three times or 10 times as much NaCl as sucrose was used an osmotic value of 4.5 atm. was superior to lesser values, and when 30 times as much NaCl as sucrose was employed the "optimum" shifted to 15 atm. Growth at this osmotic value was, however, only one-fourth that obtained at one-tenth that value in the absence of NaCl. It is noteworthy that this tenfold increase in "optimal" osmotic value was concurrent with only a threefold decrease in sucrose concentration. Satisfactory results were obtained only when the sucrose concentration was between 55 and 66 mM. The result (growth index=25) at 15 atm. and a sucrose-to-NaCl ratio of 1:30 was of the same order of magnitude as those (growth indices=26 and 20) obtained at 0.45 atm. and corresponding ratios of 1:0 and 100:1. The 33-fold difference in osmotic value produced no significant difference in growth indices. The significant feature of the solutions was not this wide range in osmotic value but rather the fact that the solutions represented closely similar sucrose concentrations of 20, 18, and 17.8 mM. This, as the table shows, is the minimum sucrose concentration capable of supporting significant growth over a one-week passage. The optimal and minimal requirements as regards sucrose are rather narrowly delimited. Osmotic value *per se* is relatively unimportant.

It is perhaps significant that when the sucrose: NaCl ratio was less than one, as it is in all animal tissue-culture nutrients, the "optimal" osmotic value likewise approached that of animal nutrients [4.5 atm. for sucrose:NaCl ratios of 1:3 or 1:10; 4.4 atm. for Baker's perfusion solution (Parker, 1938) having a corresponding ratio of 1:7]. It is clear from these results that this "optimal" value for plant tissues corresponds with the isotonic value only under special and not entirely satisfactory conditions from the point of view of capacity to support growth. It would be interesting to know whether a like condition may not hold as regards animal tissues and whether the emphasis commonly laid on the isotonicity of nutrient and perfusion fluids may not perhaps result in the use of solutions deficient in essential carbohydrate.

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NUTRIENT DEFICIENCY STUDIES AND AN IMPROVED INORGANIC NUTRIENT FOR CULTIVATION OF EXCISED TOMATO ROOTS

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Ever since the demonstration in 1934 (White, 1934) that excised plant roots can be maintained in an active state of growth for unlimited periods in a nutrient of, for the most part, known constitution, one of the major trends in plant tissue culture research has been toward the further elucidation and improvement of the constitution of this nutrient. These studies have resulted in the establishment of a completely synthetic nutrient capable of supporting a high level of growth. At several points in the development of the nutrient it has been improved by modifying either the character or the relative concentrations of certain constituents. Although a truly "optimal" nutrient could be arrived at only by the simultaneous varying of *all* constituents of the complex, which would require n^{n-1} permutations or about 2×10^{14} simultaneous sets of cultures, an approach to an optimum can be arrived at through a series of approximations: by varying one constituent, determining its optimum in the presence of a *satisfactory* concentration of all other constituents, using that provisional optimum as a basis against which to vary another constituent, and so on. The point at which this procedure ceases to give results commensurate with the effort expended must be decided by the investigator. It has seemed desirable to reexamine the solution employed in this laboratory in this manner once more before proceeding with its use.

The nutrient solution used as a standard from 1930 to date contains four categories of substances: (*a*) a solvent, water; (*b*) a carbohydrate, sucrose; (*c*) a series of inorganic salts; (*d*) certain accessory organic materials.

This nutrient was originally evolved by taking the nutrient salt mixture recommended by Uspenski and Uspenskaja (1932) for *Volvox minor* and *V. globator* and adding to it dextrose as recommended by Robbins (1922*a*) for roots of *Zea mays*, and a yeast extract adapted from the organic constituents recommended by Robbins (1922*b*) and

by Kotte (1922) for excised roots (White, 1932, 1933). The salt mixture was subjected to preliminary examination, using wheat roots as test material, and was modified by replacing K_2CO_3 with KCl and by reducing the concentration of KH_2PO_4 (White, 1933). The dextrose was subsequently replaced by sucrose (White, 1934, 1939*c*), and the yeast extract was replaced by thiamin (Bonner, 1937; Robbins and Bartley, 1937; White, 1937), glycine (White, 1939*a*), pyridoxine (Robbins and Schmidt, 1939*a*, 1939*b*; White, 1943*b*), and nicotinic acid (Bonner and Devirian, 1939; White, 1943*b*). The solution was further modified by the addition of salts of manganese, zinc, boron, and iodine (White, 1938). Since, however, except for this last modification, the inorganic solution had not previously been subjected to detailed study using the present standard test plant, tomato, the possibility existed that some constituent or constituents might prove to be unsatisfactory for tomato roots either as regards character or concentration.

A preliminary series of experiments was carried out in which each of the 14 nutrient ions or un-ionized substances making up the standard solution as of January, 1941, was omitted in turn from an otherwise complete nutrient. Twenty cultures were maintained through five passages in each deficient nutrient and in a control solution containing all nutrient constituents at the standard concentrations. The results of these 1600 cultures are shown in Figures 1 and 2 (compare White, 1942, 1943*a*).

On the basis of these orienting results, the nutrient constituents can be divided into four classes:

1. Those whose omission brings about an immediate and marked failure of the cultures: which are therefore to be tentatively considered indispensable and not present in the explants in any considerable quantity. Sucrose, iron, magnesium, calcium, phosphorus, and sulfate sulfur make up this class.

2. Those whose omission brings about a marked but more or less tardy failure of the cultures: which must therefore be tentatively considered indispensable but appear to be present in the explant in significant quantity. Thiamin is the only representative of this class.

3. Those whose omission brings about a considerable reduction in growth level, either immediate or gradual, but without complete failure of the cultures: which must thus be looked upon as beneficial but either present in other nutrient constituents as impurities in suboptimal

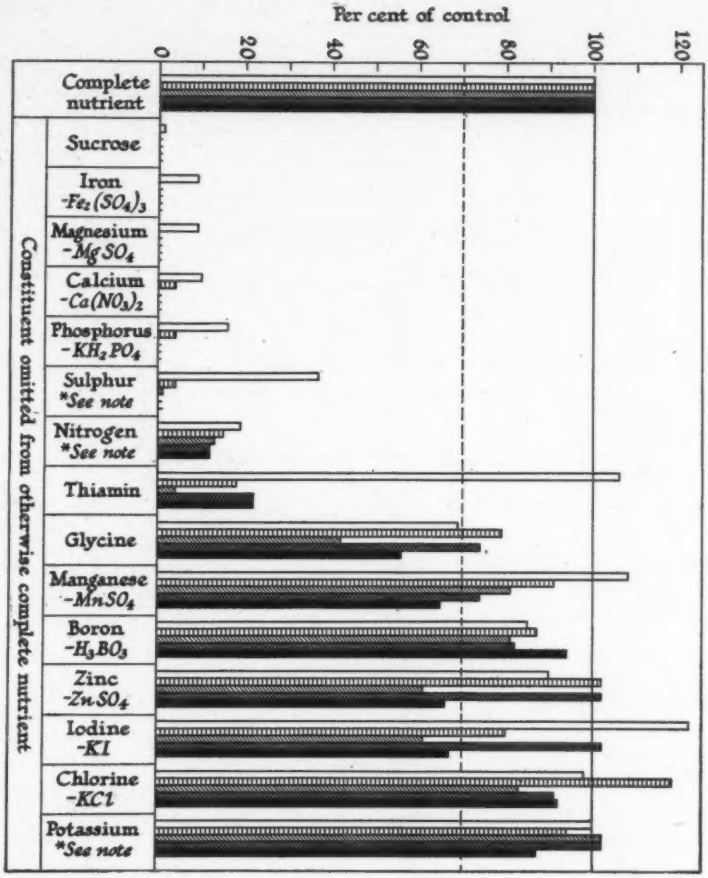

FIGURE 1

HISTOGRAM SHOWING THE EFFECTS ON GROWTH OF EXCISED TOMATO ROOTS OF OMITTING IN TURN FROM AN OTHERWISE COMPLETE NUTRIENT EACH OF THE 15 CONSTITUENTS OF THE NUTRIENT SOLUTION

Each column represents the average linear increment for one week of 20 cultures in the deficient nutrient, expressed as percentage of the increment during that same week of a corresponding group of roots in the complete nutrient. The columns for "sulfur" refer only to sulfate, since a small but undetermined amount of sulfur was included in the thiamin present in the sulfur-deficient nutrient. Those for "nitrogen" likewise refer only to nitrate, since a small but undetermined amount of nitrogen was included in the thiamin and glycine present in the nitrogen-deficient nutrient. Potassium deficiency is masked when data are recorded only in terms of linear increments (see Figure 2). (From *Pl. Physiol.*, **17**, 1942).

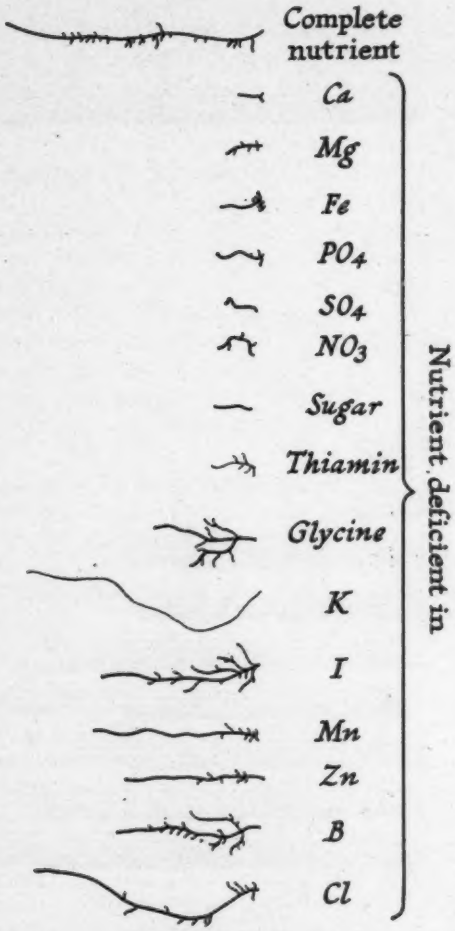

FIGURE 2

SHADOWGRAPHS OF EXCISED TOMATO ROOTS GROWN FOR FIVE PASSAGES IN EACH OF THE NUTRIENTS REPRESENTED IN FIGURE 1

The roots were chosen to represent as nearly as possible the average linear increments, branching, and general habit characteristics of those grown in each solution. X ca 0.5.

amounts or partially replaced by other materials available elsewhere in the nutrient. This class is represented by glycine, pyridoxine, and nicotinic acid and by manganese, boron, zinc, iodine, and potassium. Iodine and potassium show their essential nature chiefly in the *character* of growth obtained in nutrients deficient therein rather than in the quantitative growth values (see later).

4. Those whose omission brings about no noteworthy modification in the growth of cultures: which must therefore be tentatively looked upon as nonessential and nonbeneficial. Chloride is the only constituent of the original nutrient which seems to fall in this class. Since the chloride ion is not injurious except at relatively high concentrations, it can be used to combine with any nutrient cation whose concentration it is desired to increase.

Since every constituent of the present standard solution with the exception of chlorine can be looked upon as either necessary or beneficial to excised tomato roots, the process of determining approximate optima must be applied to them all. Sucrose (White, 1939*b*, 1939*c*), glycine (White, 1939*a*), thiamin (White, 1937), pyridoxine and nicotinic acid (White, 1943*b*) have been considered elsewhere. The "accessory salts" of manganese, zinc, boron, and iodine have also been subjected to preliminary examination elsewhere (White, 1938) and present special problems which will have to await a more extensive treatment. The present paper will deal in detail only with the concentration relationships of the ions: K^+, Na^+, Ca^{++}, Mg^{++}, Fe^{++}, NO_3^-, $SO_4^=$, and $PO^{\equiv}$.

Calcium. Calcium was present in the standard nutrient only as the nitrate. Since nitrate was also present as the potassium salt at an ionic concentration greater than that of the calcium salt, omission of the latter did not reduce the nitrate concentration enough to be likely to be, for this reason, deleterious. Calcium nitrate was present at a concentration so low in proportion to the total solute concentration that its omission did not appreciably alter either the osmotic value or the pH of the nutrient. In preparing solutions intended to have calcium concentrations below the standard, the calcium nitrate was therefore merely reduced in quantity. Solutions having calcium concentrations above the standard were prepared by adding requisite amounts of a salt in which the calcium ion was combined with the relatively inert chloride ion, i.e., $CaCl_2$. The effect of calcium concentration on growth of excised tomato roots was tested at 0.0, 0.1, 0.3, 1.0, 3.0, and 10.0 times the standard value. The corresponding per-

centage numerical growth indices at the end of three consecutive passages in these solutions were 0, 20, 43, 100, 93, and 32. In the absence of calcium the roots turned brown and the few branches which did form, not only in the first but in later passages as well, were stubby and crooked. At a concentration of 10 times the standard value the growth was likewise poor both quantitatively and qualitatively. Concentrations of 1.0 and 3.0 times the standard value did not differ significantly in effect. Under these conditions, therefore, a calcium nitrate concentration somewhat higher than the standard value of 1 mM [164 mg. $Ca(NO_3)_2$ per liter] is to be considered approximately optimal. A value of 200 mg. per liter (1.2 mM) was arbitrarily chosen as a new standard.

Magnesium. Magnesium was present in the standard solution only as the sulfate. The only other sulfates were those of iron, manganese, zinc, and iodine, present in very small amounts. Reduction in concentration of magnesium as $MgSO_4$ thus caused a simultaneous reduction in concentration of sulfate. Since in preliminary experiments no substitutions of other sulfates were carried out, the experimental results must be interpreted with some caution. Concentrations of magnesium above the standard value were obtained by addition of requisite amounts of $MgCl_2$. Experiments were carried out in which $MgNO_3$ was substituted for $MgCl_2$, but the results showed no notable advantage to be gained thereby.

Concentrations of 0.0, 0.1, 0.3, 1.0, 3.0, and 10.0 times the standard value of 0.3 mg. ions Mg were first tested. The corresponding percentage numerical growth indices after three passages were 0, 14, 49, 100, 155, and 173. Both 3.0 and 10.0 times the standard value were so far superior to the latter that a second series was carried out using concentrations of 1, 4, 8, 12, 16, 20, and 24 times the standard. The corresponding growth indices were 100, 126, 100, 98, 98, 63, and 23. The values at 1, 4, 8, 12, and 16 times the standard value thus did not differ significantly, while 20 and 24 times this value were markedly inferior for this purpose.

This last series was obtained by adding $MgCl_2$ to the basic nutrient so as not to increase the SO_4 concentration. Later experiments (see below) showed the standard concentration of sulfate to be itself suboptimal. An additional series of experiments was therefore carried out in which increased magnesium concentrations of 2, 4, 6, 8, 10, and 12 times the standard were obtained by adding $MgSO_4$ without substitu-

tion. The corresponding growth indices were 100, 111, 111, 251, 222, 154, and 98. When increased Mg content was obtained with the sulfate instead of the chloride, concentrations 6, 8, and 10 times the standard value were thus all notably superior to the latter. Since the use of the sulfate is in this case simpler than the chloride, the former was chosen for the final nutrient and was supplied at a concentration 10 times the earlier standard concentration, i.e., 3 mM (360 mg. per liter).

Iron. Omission of 2.5 mg. $Fe_2(SO_4)_3$ per liter of solution from the nutrient did not appreciably alter the latter's physical properties. Decreased iron concentrations were obtained by reduction in the amount of $Fe_2(SO_4)_3$ provided. Increased concentrations were obtained by adding $Fe_2(SO_4)_3$, $FeCl_3$, or $Fe(NO_3)_3$. All three gave like results. $Fe_2(SO_4)_3$ alone was therefore used in most experiments. Preliminary tests at 0.0, 0.1, 0.3, 1.0, 3.0, and 10.0 times the standard gave the following growth indices: 11, 173, 178, 100, 28, and 2, indicating a marked superiority of 0.1 and 0.3 times the standard concentration over the latter. Since the initial reaction was so pronounced, this and the following test were carried out for only a single passage. Tests at 0.0, 0.1, 0.3, 0.5, 0.7, 0.9, 1.1, 1.3, and 1.5 times the standard concentration gave indices of 8, 113, 164, 126, 124, 109, 99, 77, and 70, indicating again an optimum at about 0.3 the standard concentration. Subsequent tests for longer periods showed, however, that, although the linear index for a single passage was greater at 0.3 than at 1.0 times the standard, branches developed less fully and with subsequent passages the growth at the standard concentration maintained a constant level while at 0.3 times the standard it gradually deteriorated both in quantity and in quality. The original standard concentration of 6.25×10^{-3} mM $Fe_2(SO_4)_3$ was therefore retained as being, in long term experiments, very close to optimal.

The sensitivity of this chlorophyll-free tissue to iron deficiency is worthy of special note. The reaction to omission of iron was immediate. Linear increment stopped within 48 hours. Sometimes short, stubby secondary and tertiary branches formed just back of the growing point, suggesting that iron contained in the meristem might be released and re-utilized in the forming of new meristems of very brief survival, but this did not mitigate the picture of marked maladjustment. This material provides a striking demonstration of iron requirement of a nonchlorotic tissue, a distinction which is not always made clear in the teaching laboratory.

Potassium. Potassium was present in the standard solution as the nitrate, the chloride, and the phosphate. Its omission was carried out by replacing KNO_3 by $NaNO_3$, KCl by NaCl, KH_2PO_4 by NaH_2PO_4, and KI by metallic iodine. Cultures were carried in such a solution for five passages, at the end of which time the numerical growth index was 112, a value which does not represent a significant difference from the control. The roots were, however, very slender and had an average of only 4.1 branches per culture as compared to 7.5 branches per culture in the control solution. This difference was consistent and progressive, becoming more pronounced with each passage so that it can be looked upon as of real significance. The result suggests that, in the absence of potassium from the nutrient, this ion, being in the soluble form in the tissues, was passed along to the new apical meristem cells as they were formed but did not reach the older tissues, which would normally give rise to branch primordia. Branching was thus suppressed and diameter growth reduced without the linear increment being affected. It is clear that potassium is essential for normal development. But with the deficiency symptoms qualitative rather than quantitative it is difficult if not impossible to establish optimal concentration values. Information obtained in the study of the phosphate ion indicated that potassium concentration should not be greatly increased, but the experiments reported above likewise indicated that it should not be decreased markedly. The potassium concentration was therefore arbitrarily retained at the earlier standard value.

Sodium. Sodium was not present in the original solution and has repeatedly been shown not to be essential for normal development of plant tissues. It is, however, not injurious, even in considerable amounts. Studies of the sulfate and phosphate ions (to be reported later) indicated that the concentrations of both could advantageously be increased but that this must be done without a correspondingly great increase in the magnesium and potassium ions which accompanied them in the earlier solution. Na_2SO_4 and NaH_2PO_4 have therefore been added to the solution at concentrations of 1.4 mM and 0.13 mM, respectively. The data upon which these changes were based will be given under the discussion of the respective anions.

Sulfate. Sulfate was present in the basic solution as the salts of Mg, Fe, Mn, and Zn. When all of these were replaced by their respective chlorides, an abrupt and marked failure resulted (Figures 1, 2). An initial fall to about 10 per cent of the normal growth level was fol-

lowed by continued maintenance at this level, probably at the expense of the traces of sulfur contained in the thiamin and as impurities in other nutrient salts.

Concentration studies were first carried out at 0.0, 0.1, 0.3, 1.0, 3.0, 10.0, 30.0, and 100.0 times the standard values. Reduced concentrations were obtained without reduction in cation values by substituting chlorides for the salts of Mg, Mn, Fe, and Zn. Increased concentrations were obtained by adding the requisite amounts of Na_2SO_4. The numerical growth indices after three passages in these solutions were 76, 61, 97, 100, 89, 119, 123, and 7, indicating an optimum somewhere between 0.1 and 30 times the standard but probably close to the last value. A second series was therefore carried out at 0, 10, 20, 30. . . . . 90, and 100 times the standard value. The results this time were 12, 120, 116, 85, 84, 87, 79, 20, 11, 6, and 5, indicating an optimum between 10 and 20 times the standard value of approximately 0.34 milliequivalents per liter. Part of this increase was obtained by increasing the concentration of $MgSO_4$ from 0.28 mM to 2.9 mM. The remainder was made up by adding 1.4 millimols (200 mg. per liter) of Na_2SO_4, bringing the total sulfate concentration to 4.5 m equivalents per liter (including sulfates of Fe, Mn, and Zn).

Nitrate. Nitrate was present in the basic solution as salts of calcium and potassium. Substituting chlorides of these two cations resulted in abrupt decrease in growth rate to a level about 18 per cent of the normal, at which level growth continued in later passages. A trace of nitrogen was, of course, included in this "nitrate-deficient" nutrient in the glycine and thiamin and doubtless as impurities in other salt constituents. These traces of nitrogen permitted the maintenance of growth at this low level.

Concentration tests were run at 0.0, 0.1, 0.3, 1.0, 3.0, 10.0, 30.0, and 100.0 times the standard value. Reduced nitrate concentrations were obtained by substituting chlorides of calcium and potassium for the nitrates. Increased concentrations were provided by adding sodium nitrate in requisite amounts. The resulting growth indices after three passages were 3, 58, 76, 100, 106, 64, 8, and 1, indicating that the original standard concentration was very close to optimal. Since the standard calcium and potassium concentrations were likewise approximately optimal, no change was made in either nitrate.

Phosphate. Phosphate was present in the standard nutrient only as the monobasic potassium salt. Since potassium was present also as the

nitrate, chloride, and iodide, omission of the small amount of phosphate normally included did not seriously affect the cation balance. Increased concentrations of phosphate were obtained by adding requisite amounts of NaH_2PO_4. Tests were made at 0.0, 0.1, 0.3, 1.0, 3.0, 10.0, 30.0, and 100.0 times the standard concentration. The corresponding growth indices were 6, 94, 97, 100, 80, 76, 89, and 1, indicating that, while phosphate was essential for normal growth and a concentration 100 times the standard was injurious, concentrations between 0.1 and 30 times the standard value had little difference in effectiveness.

Phosphate may, however, particularly at the higher concentrations, serve both as a source of inorganic phosphorus and as a buffer. At the standard concentration of 0.13 mM this buffering action is slight but significant. To determine its effect, a series was run in which NaH_2PO_4 and Na_2HPO_4 concentrations were simultaneously varied at a constant PO_4 value in the following series: $NaH_2PO_4 : Na_2HPO_4 = 0:0$ (control 1), 10:0 (control 2), 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, and 0:10. The resulting indices were 9, 100, 98, 95, 102, 61, 36, 43, 41, 42, 39, and 39. Proportions as high as seven parts of NaH_2PO_4 to three parts of Na_2HPO_4 thus maintained normal growth but not higher proportions of the dibasic salt, nor were any of these mixtures significantly superior to the straight monobasic phosphate at the original standard concentration. Concentration of this ion was therefore left approximately unchanged, but the sodium salt was substituted for the potassium salt.

Discussion

In the experiments just presented each of the ions, K^+, Ca^{++}, Mg^{++}, Fe^{++}, NO_3^-, $SO_4^=$, and $PO_4^{\equiv}$, was systematically examined at a graded series of concentrations to determine the significance of these various levels in the nutrition of a standard clone of excised tomato roots. The ions Na^+ and Cl^- were likewise varied incidentally in connection with the other detailed studies. The results are indicated in Table 1.

The earlier standard concentrations (White, 1933) of K^+, Ca^{++}, Fe^{++}, NO_3^-, Cl^-, and $PO_4^{\equiv}$ were approximately optimal and needed to be altered only slightly. The concentration of Mg^{++}, on the other hand, was markedly suboptimal (compare White, 1933) and the concentration of $SO_4^=$ was so far below the optimum that, in order to increase it sufficiently, the sodium ion had to be introduced to carry

TABLE 1

Ion	Standard conc. = S	Optimal conc.	
	Milliequivalents per liter	x S	Milliequivalents per liter
K^+	1.8	1	1.8
Na^+	—	—	1.4
Ca^{++}	0.3	1-3	0.6
Mg^{++}	0.15	10	1.5
Fe^{++}	0.002	1	0.002
NO_3^-	1.4	1-3	2.0
Cl^-	0.9	1	0.9
$SO_4^=$	0.175	10-20	2.2
$PO_4^{\equiv}$	0.09	1-2	0.14
Total	4.817		10.542

it. We have arrived, then, by a new series of approximations, at a new standard solution having the following constitution:

$MgSO_4$	360 mg.	$MnSO_4$	4.5 mg.
$Ca(NO_3)_2$	200 mg.	$Fe_2(SO_4)_3$	2.5 mg.
Na_2SO_4	200 mg.	$ZnSO_4$	1.5 mg.
KNO_3	80 mg.	H_3BO_3	1.5 mg.
KCl	65 mg.	KI	0.75 mg.
NaH_2PO_4	16.5 mg.	H_2O	1000 ml.

It is interesting to note how closely this approximates the solution developed by somewhat cruder methods 10 years ago (White, 1932, 1933), using a different test plant. Only the magnesium and sulfate have required considerable change, and it may be recalled that evidence of the probable desirability of increasing the magnesium concentration above the value tentatively chosen was mentioned in my 1933 paper. This confirmation of the earlier conclusions is a gratifying indication of the correctness of that work.

Tests over a period of 13 passages showed this nutrient to be approximately 25 per cent better than the former standard (6.3 mm./cult./day as compared with 5.0 mm./cult./day in the earlier solution). This new inorganic solution has therefore been adopted since December 1, 1941, as a new standard.

Summary

Routine examination of the effects on growth of excised tomato roots of graded series of concentrations of all the inorganic ions of the nutrient solution with the exception of Mn, Zn, B, and I have resulted in modifications which improve the effectiveness of this solution on these roots by about 25 per cent. This improvement has resulted chiefly from a considerable increase in concentrations of magnesium and of sulfate, although concentrations of calcium, nitrate, and phosphate have likewise been slightly increased. The sodium ion, not present in the earlier solution, has been introduced as a carrier for sulfate and phosphate but without implying any *per se* significance.

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THE GROWTH ACTIVATING EFFECT OF EXTRACT OF ADULT TISSUE ON FIBROBLAST COLONIES IN VITRO: III. THE CULTIVATION FOR PROLONGED PERIODS*

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In the course of investigations (Doljanski, L., & Hoffman, R., 1939; Hoffman, R., & Doljanski, L., 1939; Hoffman, R., Tenenbaum, E., & Doljanski, L., 1939; Doljanski, L., Hoffman, R., & Tenenbaum, E., 1939; Hoffman, R., Tenenbaum, E., & Doljanski, L., 1940; Doljanski, L., Hoffman, R., & Tenenbaum, E., 1942) carried out in this laboratory during the past few years it was repeatedly shown that extracts from tissues and organs of adult animals markedly stimulate the growth of cell colonies in vitro. In the above experiments the growth promoting effect of tissue extract was followed for a limited period of seven days. In furtherance of this study, we attempted to ascertain whether cell colonies could be maintained in adult tissue extract median permanently.

A. EXPERIMENTAL PROCEDURE

1. *Tissue Extracts*

Tissue extracts were prepared from adult chicken heart muscle and from adult chicken brain. The tissue was minced finely with scissors and suspended in four times its volume of Tyrode solution. The resulting suspension was allowed to stand for 24 hours in the refrigerator, and after centrifugation the supernatant fluid was decanted. Heart extract was used in the original concentration. Brain extract was diluted before use with an equal quantity of Tyrode solution.

2. *Tissue Cultures*

Cultures were made from heart muscle of adult fowls. Heart muscle fragments of uniform size were planted in Carrel flasks. The medium was composed of 0.5 cc. adult fowl plasms diluted with 1 cc. Tyrode solution. Two drops of adult heart extract were added in order to coagulate the plasma. The supernatant fluid phase was composed of

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0.5 cc. tissue extract. Every fourth day the cultures were washed in Tyrode solution and fresh tissue extract was added. At intervals the coagulum was reinforced by the addition of fresh plasma diluted with Tyrode solution. The cultures were transferred every 16-20 days. At transferring it was necessary in most cases to divide the cultures into either two or four parts. At given intervals the cultures were projected, drawn and measured according to the method of Ebeling.

B. Results

It proved possible to cultivate colonies of fibroblasts derived from adult heart muscle in media composed of adult fowl plasma and extracts of adult animal tissues continuously. The growth of fibroblast cultures in heart extract medium was followed for a period of one year. In brain extract medium the cell colonies were maintained for six months. After these periods, cultivation was discontinued. At the conclusion of the experiments the cultures showed considerable growth capacity (Figure 1); their growth rate throughout the entire period of cultivation remained undiminished (Figure 2). The cells maintained their healthy appearance. The texture of the colonies remained dense and their architecture uniform (Figure 3).

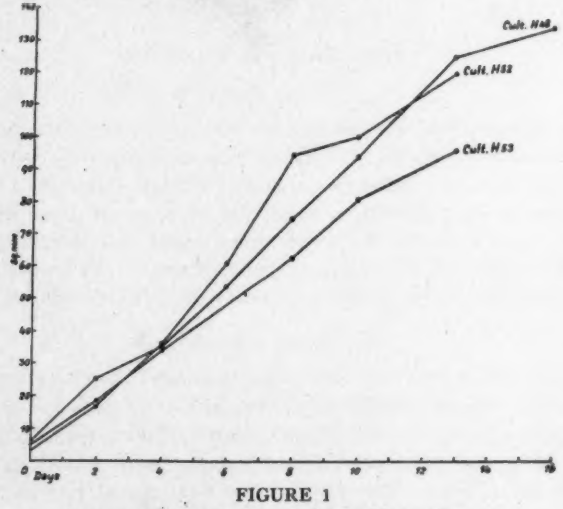

FIGURE 1

Growth of Fibroblasts in Adult Heart Extract
(Ninth passage; total period of cultivation; 7-8 months.)

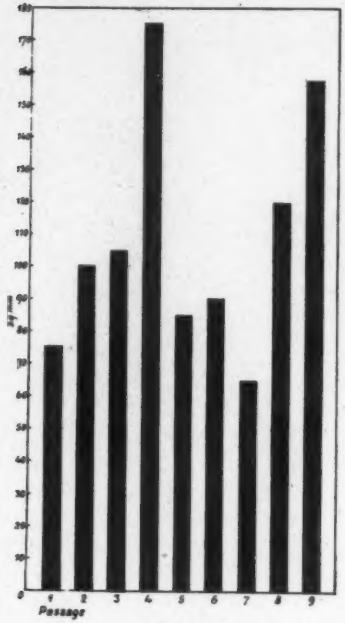

FIGURE 2

OUTGROWTH OF FIBROBLAST CULTURES IN ADULT HEART EXTRACT MEDIUM AT NINE SUCCESSIVE PASSAGES

Each vertical represents the growth area attained in a period of 16-20 days.

C. DISCUSSION

Carrel (1913) was the first to refer to the ability of extracts of adult tissue to stimulate the growth of cells in vitro. He believed, however, that there is a fundamental difference between the action on cell growth of extracts of adult tissues and that of embryonic tissues. According to Carrel and Ebeling (1923) embryonic extracts stimulate cell proliferation as well as enable prolonged growth of fibroblasts in vitro. Extracts of homologous adult tissues, on the other hand, at first determine an increase in the mass of fibroblasts cultures; after a few passages, however, the fibroblasts in this medium cease multiplying and ultimately die. "Extracts of adult tissues were not capable of maintaining indefinitely the life of the fibroblasts in vitro, as fresh

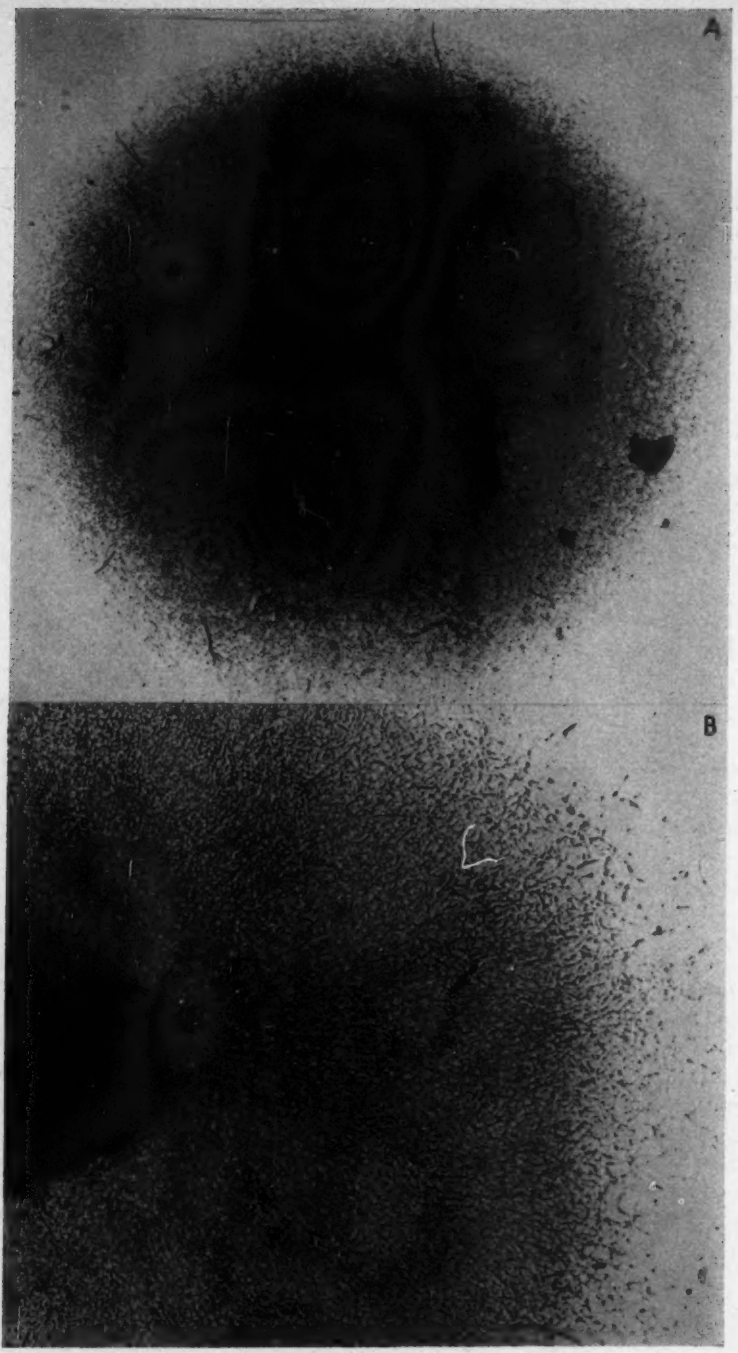

FIGURE 3

CULTURES OF FIBROBLASTS MAINTAINED THROUGH SEVEN PASSAGES (ABOUT SIX MONTH) IN ADULT HEART EXTRACT MEDIUM; LIVING
a: Mag. x 9; *b*: Mag. x 17.)

embryonic juice does." Carrel (1924) concluded that "fibroblasts and epithelial cells may be considered as in a true state of cultivation only when embryonic tissue juices are present in the medium."

The results reported here are in disaccord with those cited above. This contradiction, however, is only apparent. All of Carrel's experiments were carried out in hanging drop cultures. Under these conditions Carrel's statement is fully valid. Despite repeated efforts we were likewise unable to maintain fibroblasts for prolonged time periods in hanging drop cultures in adult tissue extract medium. In the first few passages the cultures developed quite satisfactorily, their area of growth being considerable, dense in texture, and composed of healthy cells. After subsequent transferring, however, the growth becomes more sparse and after six or seven passages further cultivation failed.

The results obtained when the flask method is used for cultivation of all colonies are quite different. As has been shown in the present paper, fibroblast colonies grow well in a medium composed of adult plasma to which either adult heart or brain extract is added. These colonies may be transferred without difficulty from flask to flask. Their growth activity does not diminish in the course of transferring, nor is the health of the cells affected throughout prolonged cultivation.

From these experiments it is evident that adult cells may be cultivated outside the organism permanently and without diminution in growth capacity in a medium completely free of embryonic extract and composed entirely of adult plasma and adult tissue extract.

The explanation of the discrepancy between Carrel's statement and the conclusions drawn by us must be sought in the fact that cells maintained respectively in hanging drops and Carrel flasks live in conditions which differ in many respects, and that the same growth stimulating factor can act differently in different environments. Experimental data which support this view will be presented in a subsequent paper.

D. Summary

It is shown that prolonged cultivation of cells in a medium composed of adult plasma and adult tissue extracts is easily realizable. Fibroblasts from adult chicken heart were cultivated in extract of adult heart muscle for a period of one year, and in extract of adult brain for a period of half a year, without decrease in their growth rate.

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THE EFFECTS OF l-TYROSINE, l-PHENYLALANINE, AND l-ARGININE UPON THE DIFFERENTIATION OF FERTILIZED TUBAL RABBIT OVA*

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The physiological effects of l-tyrosine and l-phenylalanine on the rate and quality of pulsation in the 72-hour extirpated chick heart have been reported in a previous paper (1). The growth effects of these amino acids plus the amino acid l-arginine on 48- to 96-hour fertilized tubal rabbit ova are here reported.

The adaptability of these organisms to studies concerning growth patterns and metabolism have been dealt with in a previous paper (2). Suffice it to say, the observation of fertilized ova in vitro, subjected to various alterations in their chemical environment, appears to be a good method of studying the growth problem as it applies more specifically to the mammal.

From Hammett's work (3) and that of Gudernatsch (4) the naturally occurring amino acids and certain nuclear compounds are intrinsically concerned with intracellular metabolism, appearing as it were, that each characteristic of growth is predetermined and determined by essential chemical reactions involving naturally-occurring compounds.

It has been our intention to observe the effects of these determinants of growth on the development of the earliest multipotent cell as it exists in the mammalian embryo.

METHOD

Ova were recovered from the Fallopian tubes of rabbits about 48 hours after copulation by the usual flushing out technique (2). During the course of this series of experiments and from the experiments of others, it was found that .25 to 1 cc. of solution of anterior pituitary extract, injected intravenously, produced a more abundant yield of ova. These ova were recovered and then cultured either in the circulation apparatus or D3 Carrel flasks.

Pooled serum from several does or from the same doe from which

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the ova were recovered, mixed with varying amounts of Tyrode's solution and amino acid was used as a medium in these experiments (Figure 1). Blood was obtained by heart puncture. The syringe was first rinsed with cold sterile Tyrode's solution to minimize hemolysis, the slightest amount of which is inhibitory to development. The clot was then permitted to retract at 20°C. Serum was obtained several hours later by first withdrawing the supernatant serum, followed by centrifugation.

The animals were anesthetized with ether and the Fallopian tubes excised. About 2 cc. of serum or a mixture of Tyrode's solution and serum is sufficient to wash out all ova, provided that sufficient mesosalpinx has been removed to prevent buckling of the tube during flushing. The ova were recovered by sterile pipettes and transferred to experimental conditions.

All compounds were weighed into sterile weighing bottles and then maintained at 56°C. for an hour.

Sufficient sterile Tyrode's solution, adjusted to pH 7.4, was added to weighing bottles containing sufficient amounts of amino acid to make a M/100 solution. In the case of tyrosine it was necessary first to dissolve the amino-acid in a predetermined quantity of approximately 0.1N HCl and then adjust it to pH 7.0 by an equivalent amount of base in Tyrode's solution in order that the osmotic pressure be uniformly maintained throughout the entire series. With arginine it was necessary to use the hydrochloride.

Ova ready to be studied histologically were removed from culture, washed in Tyrode's solution and dropped into Allen's modification of Bouin's fluid. They were dehydrated by the drop method with two changes of 95 per cent and absolute alcohol preceding clearing with cedar oil. Two changes of cedar oil are advantageous and they are permitted to remain in the last change of cedar oil for 24 hours. Three changes of paraffin were used. Eggs were kept under direct observation during dehydration and infiltration. This was done by using small embryological dishes placed on a hot plate during infiltration and constantly observing them with a long arm binocular microscope.

OBSERVATIONS

The following experiments were performed with ova recovered from does No. RS1, RS4, RS5, RS6, 8, 36, and 45, cultured in M/2000,

Animal no.	Age of ova at time of recovery	No. of controls	No. of exper. ova	Medium	Period of cultivation
RS_1	53 hrs	2	2	7 c.c. M/30,000 l-Tyrosine in serum	78 hrs.
45	48 hrs	2	4	7 c.c. M/2000 l-Tyrosine in 3:1 serum-Tyrode	96 hrs.
			4	7 c.c. M/10,000 l-Tyrosine in 3:1 serum-Tyrode	
36	52 hrs	2	2	7 c.c. M/20,000 l-Tyrosine in 3:1 serum-Tyrode	96 hrs.
			2	35 c.c. of above mixture in a circulating culture	
RS_5	48 hrs	3	8	35 c.c. M/100,000 l-Tyrosine in 3:1 serum-Tyrode	24 hrs.
RS_6	48 hrs	1	2	5 c.c. M/100,000 l-Tyrosine in 3:1 serum-Tyrode	48 hrs.
RS_4	48 hrs.	2	3	5 c.c. M/10,000 l-Tyrosine in 3:1 serum-Tyrode	72 hrs.
				5 c.c. M/100,000 l-Tyrosine in 3:1 serum-Tyrode	
8	24 hrs.	2	4	7 c.c. M/2,000 l-Tyrosine in 3:1 serum-Tyrode	72 hrs.
26	74 hrs.	4	4	7 c.c. M/50,000 d-Alanine in 3:1 serum-Tyrode	72 hrs.
			4	7 c.c. M/50,000 l-Arginine HCl in 3:1 serum-Tyrode	
12	48 hrs.	1	3	5 c.c. M/10,000 d-alanine in 3:1 serum-Tyrode	72 hrs.
			4	8 c.c. M/50,000 d-alanine in 3:1 serum-Tyrode	
RC_8	48 hrs.	2	2	6 c.c. M/50,000 Arginine-HCl in 3:1 serum-Tyrode	72 hrs.
			2	6 c.c. M/50,000 d-Alanine in 3:1 serum-Tyrode	
RC_7	48 hrs.	1	2	8 c.c. M/10,000 Arginine HCl in 3:1 serum-Tyrode	96 hrs.
			2	8 c.c. M/50,000 Arginine HCl in 3:1 serum-Tyrode	
18	48 hrs.	2	3	8 c.c. M/10,000 Arginine HCl in 3:1 serum-Tyrode	96 hrs.
			2	8 c.c. M/50,000 Arginine HCl in 3:1 serum-Tyrode	

FIGURE 1

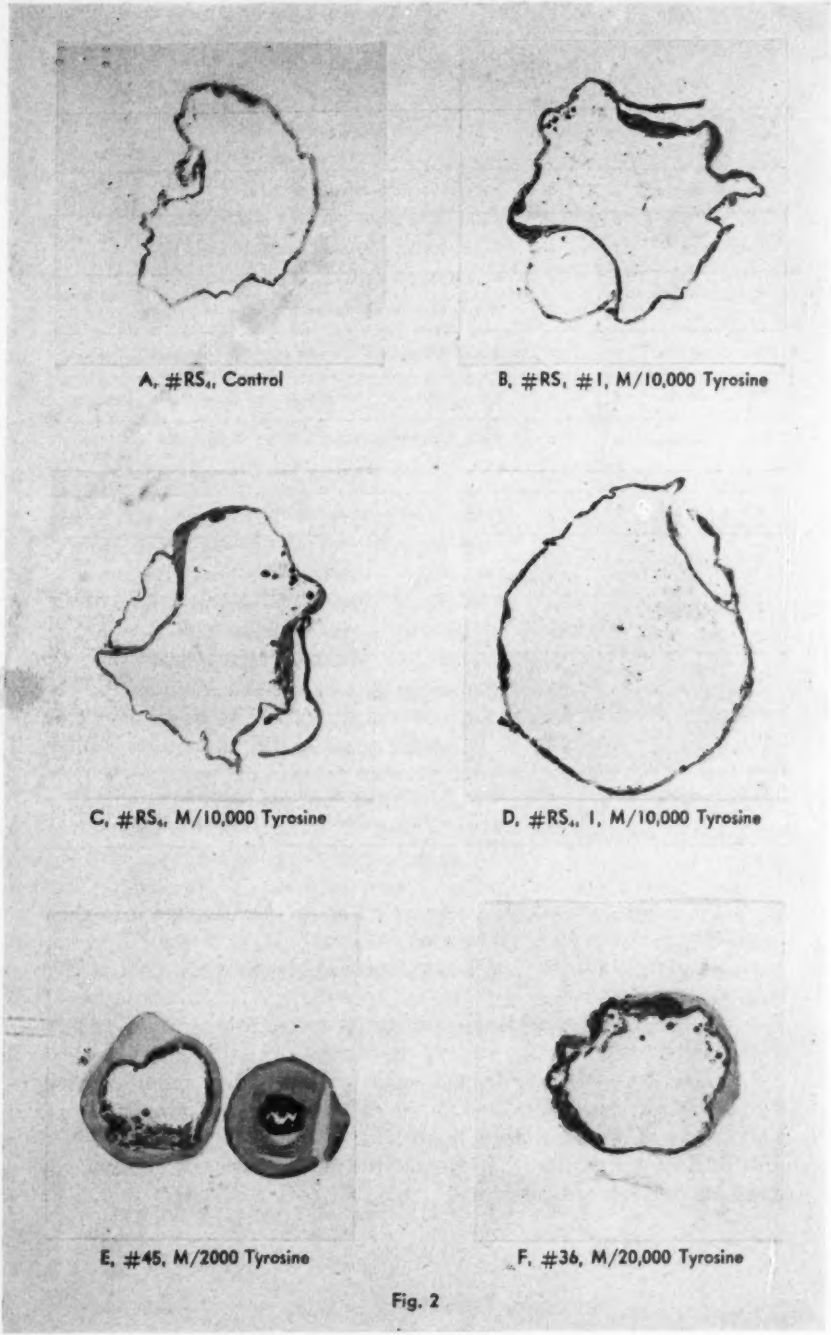
M/10,000, and M/100,000 l-tyrosine (Figure 1). Compared to their respective controls, they differed as follows:

1. Experimental ova were comparatively larger than control ova from the same animal.

2. 1/10,000 tyrosine is the optimal concentration, while 1/2000 is definitely toxic.

3. The cells of experimental ova were smaller, more regular, stained darker, and appeared more differentiated (Figure 2, *b*, *f*). There were also more mitotic figures.

4. All experimental ova presented the morphological appearance of comparatively older blastocysts.



A, #RS$_4$, Control

B, #RS, #1, M/10,000 Tyrosine

C, #RS$_4$, M/10,000 Tyrosine

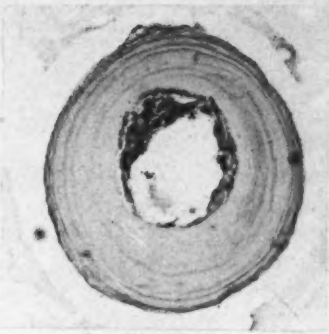
D, #RS$_4$, 1, M/10,000 Tyrosine

E, #45, M/2000 Tyrosine

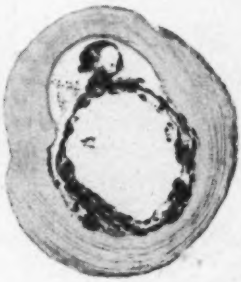
F, #36, M/20,000 Tyrosine

Fig. 2

A, #36, 2, M/20,000 Tyrosine

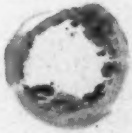


B, #RC_7, 12, Control

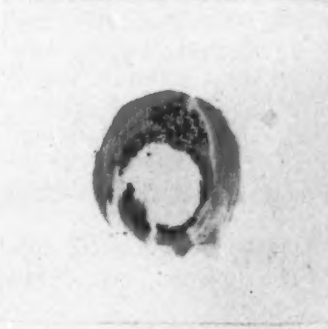


C, #12, M/50,000 Phenylalanine

D, #12, 2, M/50,000 Phenylalanine

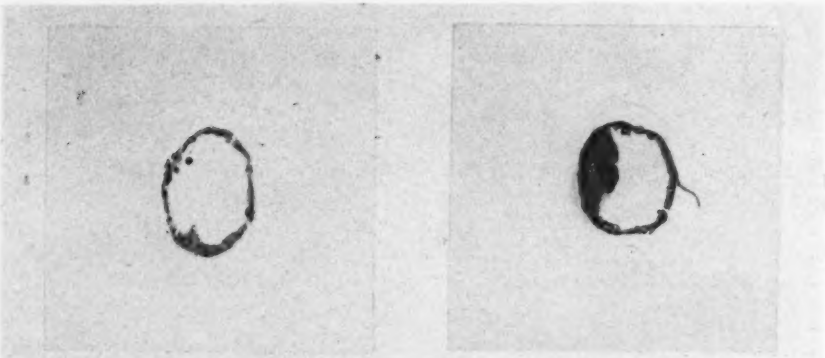


E, #RC_7, M/50,000 Arginine



F, #26, Control

Fig. 3



A, #26, M/10,000 Arginine B, #26, M/50,000 Arginine

Fig. 4

5. Experimental ova showed a number of highly basophilic round cells in close relation to trophoblast cells. This was interpreted as a manifestation of new growth production from multipotent cells. In many cases these cells were seen differentiating from trophoblast cells (Figure 2, *c, f*; Figure 3, *a*).

6. The trophoblast cells were more squamous like; again, evidence of accelerated differentiation.

7. Ova of Doe 36 showed double trophoblast formation (Figure 2, *f*).

This was not uniformly observed in all experimental ova. The following experiments were performed with ova recovered from Does 26, 12, RC8, cultured in M/10,000 and M/50,000 l-phenylalanine. Compared to their respective controls, they showed:

1. Experimental ova were uniformly larger than control ova, but they did not attain the size of ova cultured in l-tyrosine for the same period of time.

2. Differentiation was not as great as that seen with l-tyrosine.

3. There was marked doubling of the cells of the trophoblast, leading in most cases to a completey formed additional trophobast-like layer (Figure 2, *c, d*).

4. No small round cells were differentiated.

The following experiments were performed with ova recovered

from Does 26, RC8, RC7, 18, and cultured in M/10,000 and M/50,000 arginine. Compared with their respective controls they showed:

1. No increase in size over respective controls.
2. Grossly, ova had differentiated to the blastocyst stage, but were smaller than control blastocysts.
3. There were fewer cells in these ova and the cells were of much larger size, more regular, and of a more undifferentiated appearance.
4. There was no evidence of increased number of mitoses as was observed with l-tyrosine, l-phenylalanine and controls.
5. There was no evidence of round cell production as was observed with tyrosine and l-phenylalanine.
6. In general, it appeared as though these ova were inhibited in differentiation. New growth from multipotent structures was retarded.

Discussion

There was acceleration of "growth"[1] in ova cultures in l-tyrosine solution. This manifested itself by an increase in size of experimental ova over that of their respective controls.

Cytologically, experimental ova show some differences in cell type. There could be seen moderate proliferation of round cells from the trophoblast layer. These cells were not of any specific type as was the case with the amino acids l-proline, l-hydroxyproline, and aspartic acid (2). In other instances there was well defined reduplication of the trophoblast layer.

From this it appears that the effects of tyrosine are twofold. First, there is stimulation of the natural growth pattern, apparently exerting itself as a stimulant to intracellular metabolism. Secondly, in referring to the formation of round cells, l-tyrosine has some feeble ability to cause new growth from multipotent anlagen cells, as is seen with hydroxyproline, proline, and aspartic acid which have been shown by Hammett (5) and others to be specifically concerned with the initiation of new growth from embryonic anlagen cells. Experiments with Obelia (6) indicate an accentuation of this effect over that seen with mammalian ova. l-Tyrosine retards new growth production from anlagen.

l-Phenylalanine stimulates "growth" of ova, but here the effect was

[1]For discussion of the word *"growth,"* see Hammett, F. S., *Growth*, 1942, **6**, 59-123.

less marked than with l-tyrosine. However, its ability to cause reduplication of the trophoblast layer was more marked while few round cells were seen.

With the exception of the fact that the trophoblast layer was uniformly doubled, blastocysts obtained with l-phenylalanine were in accord with their normal degree of development. As is the case with l-tyrosine, the uniform acceleration of "growth" over that of controls is again a reflection of a change from a more undifferentiated cellular structure to a more specialized structure, by stimulation of intracellular metabolism. The more consistent doubling of the trophoblast layer and greater repression of round cell proliferation, as compared to l-tyrosine, indicated that l-phenylalanine has a greater retarding effect on new growth production from anlagen but stimulates new growth of less potential cells to a greater degree than does l-tyrosine.

Ova cultured in arginine had not attained the degree of differentiation, nor size, observed in controls. These ova presented the startling picture of having been stimulated structurally to blastocyst formation and retarded in cytological differentiation.

The retarding effect upon differentiation from multipotent cells has been observed in Obelia geniculata (7). The cells of the inner mass and trophoblast layers were larger and more irregular than controls.

The production of abundant non-specific round cells was reflection of the ability of arginine to stimulate proliferation and differentiation of new growth from embryonic anlagen as was observed by Hammett.

Older ova are not so susceptible to the effects of arginine as are younger ova. From this it may be safe to assume that the effects of arginine are confined to embryonic tissues which have not differentiated at all. It also appears capable of regulating the mode of differentiation of tissues.

The cells of the inner cell mass and trophoblast were larger and more embryonic than would be expected if the size of these ova were to serve as an index of development.

The next logical step would appear to be culturing ova in a mixture of the proliferation-enhancing sulphydryl group, the differentiation-enhancing acids of the proline group, and the arginine, which apparently retards differentiation, but enhances "new" proliferation.

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SOME VARIATIONS IN BASAL METABOLIC LEVELS OF RATS*

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A. INTRODUCTION

It has been shown by Hatai and Donaldson (Donaldson, 1924) that the weight of the thyroid gland of the laboratory albino rat is relatively smaller for a given body length than that of the wild gray Norway rat from which the albino variety was derived. Donaldson collected extensive dissection data up to the time of his death in 1938. An examination of these data (Keeler and King, 1942) shows a similar reduction in the relative size of the thyroid to accompany several coat color genes that happen to be present in most laboratory albinos. One of these genes is the black gene.

Observations of behavior (Keeler, 1942) revealed variations in the response to given situations. Those variations which accompanied the presence of the black (non-agouti) gene are quite outstanding and are oriented in the direction of sociability and tameness as well as a reduction of activity when compared with the wild gray coat color variety. It appears most probable that these are pleotrophic effects of the black gene rather than manifestations of genes residing in the same chromosome with the black gene.

This reduction in the weight of the thyroid accompanied by a reduction in activity obviously suggests an alteration in thyroid function as the mechanism by which the black gene modifies behavior because the association of thyroid hypofunction and reduced activity is well known. The present experiments were undertaken to determine the basal metabolic levels (average basal metabolism for each variety) for rats of several coat color constitutions and to learn whether any correlation existed between the size of thyroid or coat color and the basal metabolism. Heart rate and blood pressure were also studied since they frequently exhibit changes associated with variations in thyroid function.

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B. Materials

The rats studied consisted of 12 Wistar albinos, 14 F_2 hybrids of assorted coat colors derived from a cross between Wistar albinos and gray Norways (see Figures 1 and 2), six animals of assorted coat colors

FIGURE 1
Adult Male F_2 Hybrid Piebald Gray Rat. Note Piebald Markings on Tail

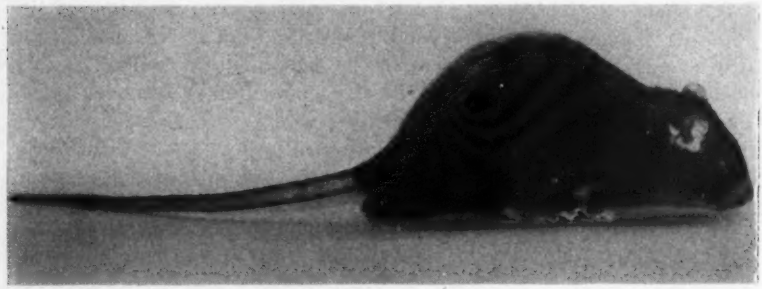


FIGURE 2
Adult Male F_2 Hybrid Gray Rat

segregating for black and hooded, four hairless albinos, and three curly hooded piebald blacks.

C. Apparatus

The metabolism apparatus was essentially that described by Schwabe and Griffith (1938). It consists of a closed system containing oxygen at atmospheric pressure. Any carbon dioxide exhaled by the animal is

quickly absorbed by a solution of barium hydroxide so that the oxygen consumed by the animal tends to cause a reduction in the pressure. The decrease in pressure is never realized, for water siphons over from the manometer system to reduce the volume in which the gas must act. A writing point floated on the surface of the water manometer produces a permanent record of the volume change upon the kymograph.

To this Schwabe-Griffith apparatus there was added a Wheatstone bridge which permitted conductrimetric titration of the barium hydroxide at any instant without necessitating the stopping of the apparatus or the removal of any of the solution. This bridge (Griffith, 1940) has two platinum electrodes immersed in the barium hydroxide solution of the set of bulbs which are opened to the animal chamber during the experimental run. By use of a 1000 cycle microhummer and earphones the conductivity could quickly and accurately be obtained.

The systolic blood pressure was measured using the indirect method upon the tail (Byron and Wilson, 1938) (Williams, Harrison and Grollman, 1939) in which the circulation of the tail is abruptly arrested by inflating an air cuff about the root of the tail. The pressure in this cuff is gradually reduced to the systolic level at which point blood is able to flow into the tail but is unable to leave. This results in an increase in the size of the tail which is detected by a water plethysmograph. Minor modifications were introduced by using glass cuffs throughout and a 25 cc. syringe instead of a leveling bulb. Beer cans with a tapered neck and with the bottoms removed proved excellent animal holders during these experiments.

The heart rate was measured by an ordinary hospital electrocardiogram machine fitted with miniature cuffs.

D. Method

Adult male rates (Davis and Hastings, 1934) were placed on the standard laboratory diet and not tested for at least a week after arrival. Measurements were repeated at intervals of about a week to obtain a check on the first values. The afternoon before the animal was to be tested all food was removed from the cage and the rat allowed to fast for at least 17 hours before the measurement of the metabolism. All experiments were performed between 10 A.M. and 4 P.M. to eliminate any diurnal variations (Horst, Mendel, and Benedict, 1934).

Since great differences in metabolic rates have been demonstrated between such seemingly similar conditions as dozing and sleeping

(Davis and Hastings, 1934), it was deemed necessary to place all animals under identical conditions. Light anesthesia produced by subcutaneous injections of Nembutal (sodium pentobarbital) 36 milligrams per kilogram body weight (Barlow and Gledhill, 1933) proved very satisfactory (Griffith and Farris, 1942). About 20 minutes after the injection the animal became totally relaxed and in this condition was placed into the glass animal chamber and after several minutes the experiment was begun. About one hour after the injection the rat began to arouse, slowly regaining normality with no noticeable after effects. That this anesthetic lowers the oxygen consumption only slightly (Chen, Anderson, and Leake, 1930) was substantiated by our results with the Wistar albinos as compared with standard values. Fresh solutions of Nembutal, concentration 10 mg. per cc., were prepared daily. The dosage as well as the surface area (Lee, 1929) was calculated from the weight of the animal.

The kymograph furnished a permanent record of the oxygen consumption rate from which the basal metabolism was calculated. The conductivity of the barium hydroxide was measured before and after the experiment and from these values the volume of carbon dioxide exhaled was computed. The respiratory quotient, obtained from the CO_2 and O_2 volumes, served as a check upon the correct functioning of the apparatus, results only being accepted when the quotient was about 0.72.

Immediately upon completion of the metabolism measurement the still drowsy animal was transferred to the warming box where it was subjected to a temperature of 40°-42°C. for 10 minutes. The beer can animal holder mentioned above was also placed in the warming box to reduce heat losses as much as possible when the rat was later placed into it. After this warming period which produces vasodilation the animal was arranged in the holder so that the nose protruded through the mouth of the can enabling it to breathe freely. The tail was threaded through the pressure cuff and plethysmograph following which the systolic blood pressure was determined as described above. The first reading was taken rapidly to obtain an approximate value after which a second accurate reading was made. This accurate value was then checked by a second person to reduce errors. All of these readings should be taken as rapidly as possible to eliminate any variations due to changes in body temperature. Unanesthetised animals may be used if they are trained to remain quiet in the holder, but we found such

values to differ but slightly from those obtained upon anesthetised animals.

The heart rate was determined by electrocardiography employing the same machine and method as for human work but with miniature electrodes adapted to the rat's legs. Again, anesthetised animals were used to keep the observational conditions uniform and to prevent distorted results due to any excitement.

E. Calculations and Results

The volume of oxygen consumed by the animal must be corrected to standard temperature and pressure before the metabolism can be calculated. The calorific value of oxygen used here is 4.702 Calories per liter (Benedict, Horst, and Mendel, 1932). This value is about three per cent lower than the value used for human metabolism, but is more accurate for work with rats.

The specific conductance of a cell is known to depend upon the size and character of the electrodes. Since the size of the electrodes is rarely exactly one square centimeter, and since the characteristics of platinum-blacked plates may change slightly with use, a cell constant must be determined and checked frequently using 0.01 demal potassium chloride solutions (Parker and Parker, 1924). The specific conductance of the barium hydroxide solution can be calculated from its resistivity and, after correcting for temperature variations, the normality of the solution can be determined. The temperature correction for this apparatus and the temperature range of these experiments was 200 $k_0(28°—T°)$ where k_0 is the observed specific conductance. From the normality (Leopold and Luck, 1933) and the volume of barium hydroxide in the absorbing bulbs, the amount of carbon dioxide excreted may be calculated.

The results are arranged to show the experimental results and the metabolisms derived from these data. They are grouped according to coat color characteristics and breeding (Figure 3, Table 1, Figure 4).

F. Discussion

A consideration of Figure 1 shows a definite variational trend between the coat color groups. This is surprisingly oriented in the direction opposite from that of thyroid size and degree of activity. In both the inbred and hybrid populations the gray rats are operating at a lower metabolic level than are the blacks of the same stock. The piebald

Oxygen Consumption and Metabolism

1 mm. rise of kymograph pointer = 1.234 cc. Oxygen

$$\text{Oxygen at } STP = 1.234\ (\Delta h) \times \frac{P}{760} \times \frac{273.1}{T} \text{ cc}$$

Calorific value of Oxygen = 4.702 Calories per liter

$S = 12.54\ Wt^{0.00} \times 10^{-4}$ square meters

$$\frac{BM}{S} = \frac{\text{cc. } O_2}{1000} \times 4.702 \times \frac{1440 \text{ mins.}}{t} \times \frac{1}{S} \quad \text{Calories per day per square meter}$$

Carbon Dioxide Excretion and Respiratory Quotient

$$R_c = R_s \frac{L-l}{l};\ K_c = \frac{1}{R_c};\ k = K_c C;\ k = \frac{1}{R_s} \times \frac{l}{L-l} \times C$$

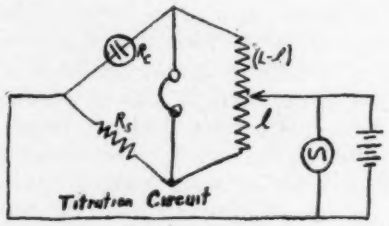
The specific conductance varies with the concentration of the solution and the amount of carbon dioxide absorbed may be calculated from the decrease in the concentration of the barium hydroxide.

milliequivalents $Ba(OH)_2$ reacting = (volume $Ba(OH)_2$ in system) $(N_f - N_o)$

1 milliequivalent $Ba(OH)_2$ = 11.207 cc. CO_2 at *STP*

$\therefore$ volume CO_2 absorbed = $(N_f - N_o)$ (volume) (11.207)

$$RQ = \frac{\text{volume } CO_2}{\text{volume } O_2}$$



Key to Symbols

STP	Standard Temperature and Pressure	C	cell constant
Δh	rise of pointer on kymograph	R_s	standard resistance
P	atmospheric pressure in mm. Hg	R_c	resistance of $Ba(OH)_2$ in the cell
T	temperature in degrees Kelvin	$\frac{L-l}{l}$	ratio of lengths of resistance wire
BM	basal metabolism	K_c	conductivity of the $Ba(OH)_2$
S	body surface in square meters	k	specific conductance
t	duration of the experiment in mins.	N_f	normality of $Ba(OH)_2$ after test
RQ	respiratory quotient	N_o	normality of $Ba(OH)_2$ before test

FIGURE 3

gene seems to have the effect of lowering the metabolic level both in the case of the black hybrids and gray inbred animals. In the black hybrids its effect in one dose (belly-spotting) and in two doses (piebald) is almost a mathematical subtraction. However, the reversal of these results in the hybrid piebald grays versus the hybrid grays must be considered and eliminated before any definite statement can be made.

TABLE 1

THE BASAL METABOLISM OF RATS WITH VARIOUS COAT COLOR CONSTITUTIONS AND THE DATA FROM WHICH IT WAS DERIVED

Rat No.	Date	Weight	1/S	T	P	Δk	t	BM	Cal./day/m²	Rat avg.	Group avg.
					Inbred Piebald Gray						593.9
1	7-28	354.4	23.55	28.2	763.3	82.1	23	27.15	639.4		
	8- 4	357.8	23.42	28.0	762.0	85.9	28	23.31	545.9		
	8-11	374.9	22.77	28.0	760.5	85.1	25	25.81	587.7	591.0	
2	7-28	300.6	26.00	28.5	763.3	84.4	27	23.75	617.5		
	8- 4	320.0	25.04	28.0	762.0	85.7	27	24.12	604.0	610.7	
3	8- 4	340.1	24.14	27.9	762.0	83.9	27	23.89	576.7		
	8-11	355.3	23.51	27.0	760.5	84.5	26	24.73	581.4	579.1	
4	7-28	270.8	27.68	29.2	763.3	60.4	23½	19.48	539.2		
	8-15	307.0	25.67	28.0	763.6	86.5	26	25.33	650.2	594.7	
					Inbred Gray						678.5
10	8- 4	264.5	28.07	28.0	762.0	79.7	25	24.22	679.8		
	8-11	282.2	27.00	27.0	760.5	85.7	26	25.08	677.2	678.5	
					Inbred Black						803.0
20	7-28	326.1	24.76	28.0	763.3	85.2	20	32.42	802.7		
	8- 4	344.1	23.97	28.0	762.0	83.8	19	33.51	803.2	803.0	
					F_2 *Hybrid Piebald Gray*						629.2
30	7-24	487.8	19.45	29.7	765.5	83.5	19	32.72	636.3		
	8- 6	481.8	19.59	27.5	762.3	88.6	21	32.12	629.2	632.7	
31	7-30	427.0	21.06	29.0	761.3	86.0	22	29.57	622.7		
	8- 7	423.4	21.17	26.5	765.3	86.2	24	27.54	582.9	602.8	
32	8- 7	455.7	20.25	27.0	765.3	84.2	21	30.70	621.8		
	8-13	465.6	19.95	27.0	764.3	88.0	20	33.64	672.6	652.2	
					F_2 *Hybrid Gray*						518.1
40	7-30	452.8	20.33	29.4	761.0	82.0	25	24.77	503.6		
	8- 7	460.9	20.12	28.0	765.3	86.9	26	26.02	523.4	513.5	
41	7-30	470.2	19.88	29.5	761.0	86.7	26	25.18	500.5		
	8- 7	467.0	19.96	27.5	765.3	89.3	25	27.30	544.9	522.7	
					F_2 *Hybrid Piebald Black*						610.3
50	8- 6	497.5	19.21	27.3	762.3	87.0	20	33.14	636.6		
	8-13	509.6	18.94	28.0	764.3	85.5	21	31.03	587.7	612.2	
51	7-22	367.4	23.05	29.3	763.5	70.8	20	26.99	622.1		
	8- 6	382.6	22.49	28.3	762.8	83.5	24	26.44	594.6	608.4	
					F_2 *Hybrid Spotted Black*						635.2
60	8- 6	401.6	21.85	28.0	762.8	87.6	23	28.97	633.0		
	8-13	417.2	21.36	27.0	764.3	84.7	22	29.44	628.8	630.9	
61	7-10	399.2	21.93	29.1	763.9	89.1	23	29.41	647.2		
	7-22	381.9	22.52	29.1	763.5	74.9	19	28.06	631.9	639.5	
					F_2 *Hybrid Black*						676.3
70	7-22	331.3	24.52	29.8	763.5	84.2	23	27.70	679.2		
	8- 6	345.7	23.90	27.3	762.3	87.6	24	27.81	664.7	671.9	
71	7-10	324.4	24.83	28.5	765.3	81.8	23	27.09	672.6		
	7-22	315.6	25.25	29.7	763.2	25.1	5¾	27.27	688.6	680.6	
					Curly Hooded Piebald Black						702.3
80	7-16	294.8	26.30	30.2	764.3	72.4	20	26.91	707.7		
	7-29	312.8	25.38	29.6	761.4	81.0	23	26.59	674.9	691.3	

TABLE 1 (*Continued*)

Rat No.	Date	Weight	1 S	T	P	Δh	t	BM	Cal./ day/m^2	Rat avg.	Group avg.
81	8- 5	315.4	25.26	28.0	764.4	85.9	23	28.47	719.2		
	8-11	335.6	24.33	28.0	760.5	86.0	22	29.64	721.1	720.1	
82	7-14	295.7	26.25	30.0	760.3	86.1	24	27.01	709.0		
	7-16	287.5	26.70	30.4	764.3	83.1	20	25.55	682.2	695.6	
					Wistar Albino						706.1
90	7- 7	320.0	25.04	28.6	760.1	86.6	24	27.32	684.1		
	7-14	343.1	24.01	31.9	759.6	82.1	21	29.22	701.7		
	7-17	335.9	24.32	31.0	765.7	87.9	22	30.20	734.5	706.7	
91	7- 8	240.8	29.70	29.3	757.8	81.3	27	22.65	672.6		
	7-21	247.8	29.19	32.3	760.5	81.9	28	21.88	638.7	655.7	
92	7- 3	332.8	24.46	27.5	762.7	84.8	21½	30.04	734.7		
	7- 8	338.0	24.23	29.4	758.1	81.1	19	32.11	778.0	756.3	
93	7- 7	404.3	21.76	29.2	760.1	84.2	18	35.31	768.4		
	7-17	420.2	21.26	30.1	766.5	84.5	18	35.63	757.6	763.0	
94	7- 7	356.2	23.48	29.7	760.1	83.6	22	28.64	672.4		
	7-17	371.9	22.88	31.0	765.7	88.1	22	30.27	692.6	682.5	
95	7- 7	324.2	24.84	28.5	760.1	76.9	22	26.45	657.0		
	7-21	343.0	24.02	32.0	760.5	83.3	23	27.10	650.9	654.0	
96	7- 7	343.1	24.01	29.8	760.1	84.5	21	30.31	727.8		
	7-17	359.2	23.36	30.0	767.0	84.8	21	30.68	716.7	722.3	
97	7- 3	275.8	27.37	26.9	762.7	81.6	25	24.91	681.9		
	7- 8	271.9	27.61	28.5	758.1	84.9	24	26.69	736.9	709.4	
98	7- 3	360.0	23.33	26.9	762.7	85.4	22	29.63	691.3		
	7- 8	355.9	23.49	29.5	758.7	86.5	23	28.28	664.3	677.8	
99	7- 3	312.8	25.38	27.4	762.7	84.1	22½	28.48	722.9		
	7- 9	314.0	25.32	28.9	761.2	85.5	24	26.96	682.8	702.8	
100	7-17	418.3	21.32	31.0	765.7	83.9	18	35.23	751.1		
	7-21	404.8	21.74	32.1	760.5	88.7	21	31.60	687.1	719.1	
101	7- 3	322.8	24.91	27.5	762.7	81.8	21	29.67	739.0		
	7- 9	319.5	25.06	29.0	761.2	85.9	23	28.25	708.0	723.5	
					F_2 *Hybrid Piebald Gray—Thyroid-parathyroidectomized*						640.1
110	7-30	379.6	22.60	29.0	761.3	78.5	21	28.28	639.1		
	8- 7	389.3	22.26	28.0	765.3	86.8	23	28.80	641.1	640.1	
					F_2 *Hybrid Gray—Thyroid-parathyroidectomized*						623.8
120	7-24	369.0	22.99	29.2	765.7	67.9	20	25.35	582.7		
	8- 7	349.3	23.76	28.0	765.3	84.5	24	26.87	638.3	615.5	
121	8- 7	458.1	20.19	28.0	765.3	87.4	21	31.76	641.2		
	8-13	454.0	20.30	28.0	764.3	84.6	21	30.70	623.2	632.2	

For this reason further studies on larger numbers of siblings are being planned to eliminate the effects of extraneous genetic characteristics. In no case did the metabolism of the grays reach the level of the albino variety, which is really pure bred for black, piebald, and albinism, and only the one black inbred animal surpassed it among the black animals.

Since segregation for the Wistar albino types of metabolism (high) was not noted in the F_2 hybrids, it is probable that the inheritance of a

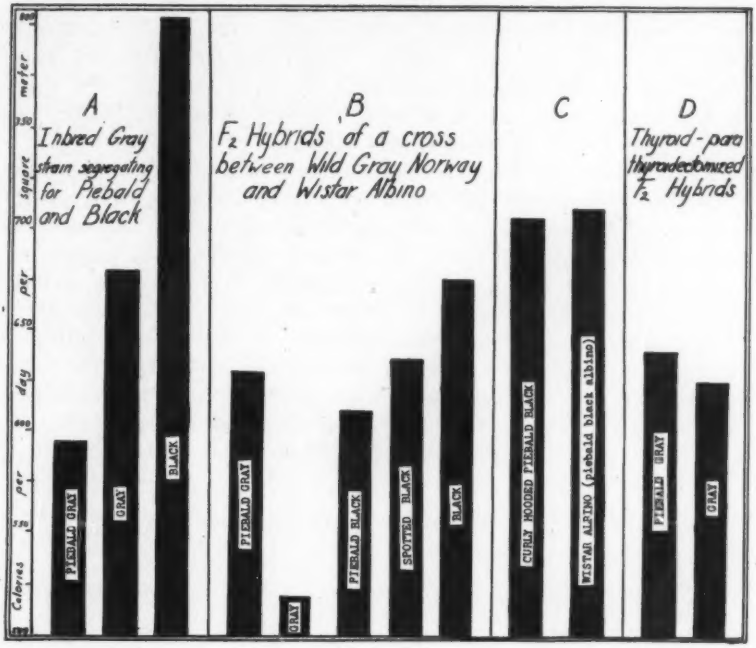


FIGURE 4

AVERAGE METABOLIC RATES OF RATS CLASSIFIED ACCORDING TO COAT COLOR AND BREEDING

high metabolism as contrasted with the low metabolism of the wild gray rat is dependent upon more than one pair of genes. If the heredity of the Wistar albino type metabolism had been dependent upon a difference of but one pair of genes in the total genetic constitution from the wild gray, one would have expected the high metabolic rate to have appeared in one out of four of the F_2 hybrids, which event did not occur.

The association of clinical hyperthyroidism with a normal or subnormal basal metabolism is not an unusual finding in humans, although the cause for this association is not known. Experimental production of hyperthyroidism through repeated injections of thyrotropic hormone (Siebert and Smith, 1929; Marine and Rosen, 1933) at first causes the basal metabolism to increase but, after a few weeks, the metabolism falls to subnormal while the thyroid hyperplasia and the nervous mani-

festations persist. That this is the true explanation here is not known, but at least the picture is the same in both cases. In these rats the possibility that this association of hyperthyroidism with low basal metabolism is the result of pleotrophic effects of the coat color genes must also be considered. At present, however, the mechanism remains unknown and must be clarified by further work.

The occurrence of normal basal metabolisms in the thyroid-parathyroidectomized animals was found to be due to hyperplasia of the thyroid. The operation had been performed without the aid of a dissecting microscope eight weeks before the measurements of metabolism were made. Although at the time of operation it was thought that all of the glandular tissue had been excised, small amounts of hyperplastic tissue with the high type epithelium were found in the histological sections prepared after sacrificing the animals. No parathyroid tissue was present but it is known that the rat can live without them, showing only a calcium deficiency (Schour, Chandler, and Tweedy, 1937).

The albino hairless rats exhibited a higher metabolic level (Table 2)

TABLE 2

THE BASAL METABOLISM OF HAIRLESS ALBINO RATS AND THE DATA FROM WHICH IT WAS DERIVED

Rat No.	Date	Grams Weight	*S*	*T*	*P*	Δh	*t*	*BM*	Cal./day/m^2
131	7-23	170.0	36.60	29.0	763.0	69.2	19	27.59	983.5
	7-29	187.8	34.47	30.6	760.4	81.3	22	27.78	957.7
	8-13	241.2	29.67	27.5	764.3	86.9	22	30.15	894.5
132	7-23	162.5	37.60	28.9	763.0	67.2	20	25.49	958.4
	7-29	176.5	35.78	30.6	760.4	83.0	26	24.00	858.7
	8- 5	202.2	32.98	28.0	764.4	83.6	25	25.49	840.6
133	7-23	267.8	27.86	29.1	762.3	70.5	17	31.46	876.5
	7-29	260.8	28.31	30.2	760.4	87.8	26	25.42	719.6
	8- 5	274.1	27.48	28.0	764.4	81.3	24	25.82	709.4
134	7-23	249.8	29.05	29.0	763.0	68.6	17	30.61	889.2
	7-29	248.9	29.11	30.4	760.4	81.2	23	27.42	798.3
	8- 5	265.2	28.03	28.0	764.3	83.7	24	26.58	744.9

than the normal hair-bearing variety as would be expected, since they have lost their insulating coat. However, an interesting trend was noted. The rats tested were 3-6 months old and although they were continually losing more and more hair, the metabolic level continuously *decreased*. The loss of hair is accompanied by the development of numerous dermoid cysts which gradually harden, fall out, and leave empty craters. The skin becomes coarse and hangs in loose folds suggesting a pock-

marked old man. It may be that this heavy, thickened skin permits less and less radiation and evaporation which processes normally account for the greater amount of heat losses. The animal would thus be required to expend less and less energy to maintain the body temperature and the metabolism would decrease.

A study of the heart rates (Table 3) also shows variations associated

TABLE 3
HEART RATES OF SEVERAL VARIETIES OF RATS

Rat No.	beats / min.	Group avg.
	Wistar albino	
94	456	
95	402	430
96	435	
100	456	
	F_2 *hybrid piebald gray*	
31	461	
32	446	454
	F_2 *hybrid gray*	
40	414	
41	360	387
	F_2 *hybrid piebald black*	
50	427	
51	440	434
	F_2 *hybrid spotted black*	
60	410	
61	420	415
	F_2 *hybrid black*	
70	393	393

with coat color. Most marked is the effect of the piebald gene which causes an increase in the rate. Again the difference between one and two doses is noted in the piebald and spotted black rats.

The measurements of blood pressure disclosed no significant differences between animals bearing the various coat color characteristics. The range lay between 110 and 140 with a mode of about 120 mm. of mercury. However, the thyroid-parathyroidectomized animals were markedly hypotensive with an average systolic pressure of only 98 mm. This may indicate that the subtotal removal of the thyroid and complete removal of the parathyroid glands exerts an effect from which the cardiovascular system requires a longer time to recover than does the general metabolism.

G. Conclusions

1. Wild gray Norway rats operate at a lower basal metabolic level than do Wistar albinos.
2. Hybrids between the wild gray Norway and the Wistar albino inherit the lower metabolism of the gray parent.
3. The most important factors in this alteration of basal metabolism are the coat color genes.
4. Because segregation for high (Wistar albino type) metabolism in the F_2 hybrids was not noted, inheritance of this type is probably based upon more than one pair of genes.
5. Our findings of high basal metabolism in the hairless albinos confirms the findings of Benedict. We also find that the metabolism decreases with age and its concomitant hair and skin changes.
6. The heart rate also varies with the coat color.
7. Blood pressure probably requires a longer period of time to return to normal than does basal metabolism following parathyroidectomy and subtotal thyroidectomy.

H. Summary

Experiments upon rats of various coat color characteristics showed variations in basal metabolism and heart rate which appear associated in some manner with the coat color. A decrease in basal metabolism with age is shown by the hairless albino rats.

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